STUDIES ON PLASMID ENCODED VIRULENCE FACTORS IN THE STRAINS OF *Escherichia coli* OF MAN AND ANIMALS: COMPATIBILITY WITH R-PLASMIDS

THESIS SUBMITTED FOR THE DEGREE OF

Doctor of Philosophy

IN

AGRICULTURE

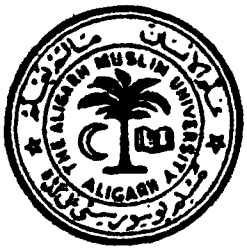
(Microbiology)

Aligarh Muslim University, Aligarh

IQBAL AHMAD

DIVISION OF MICROBIOLOGY
CENTRAL DRUG RESEARCH INSTITUTE,
LUCKNOW, (INDIA)

1993



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SUMMARY

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Pathogenic bacteria have a chemical armoury which enables them to invade a host and produce diseases. The problem is to identify the weapons in this armoury, their relative importance, chemical nature and mode of action on the host. This task is relatively simple when pathogenicity is determined by a single bacterial product, easily produced *in vitro*, as in diphtheria and tetanus. However, in majority including *Escherichia coli* infections the pathogenicity can not be related to a single microbial product and its biochemical basis are difficult to identify.

A significant development in our understanding of *E.coli* infections took place when it was discovered that the genetic determinants of certain virulence characteristics such as enterotoxins and adhesiveness could be carried by plasmids. However, the presence of plasmids is not always required for virulence nor it is the only factor involved.

Plasmids are sometime unstable and this may make it possible to relate changes in the virulence of the organism with the loss of plasmids. Re-introduction of the plasmid into the wild type and indicator recipient strains can then confirm that genes coding for a particular character carried on the plasmid.

Except for enterotoxins however, little is known about the precise role and relative importance of these virulence factors in determining the pathological potential of bacteria in the development of pathogenic conditions.

The present investigation was conducted to explore the presence of various plasmid encoded characters including drug resistance and various virulence factors (enterotoxins, colicins, haemolysins and adhering factors), their infectious nature and role in influencing the virulence

and pathogenicity of *E. coli* strains. These virulence factors have also been investigated for their affinity with drug-resistance plasmids in various combinations under natural condition. The results and conclusion drawn in the present investigation can broadly be divided under following heads.

BIOCHEMICAL AND SEROLOGICAL STUDIES:

A total of 194 strains of *E.coli* were isolated from human (UTI,30 strains; GIT, 33), monkeys (GIT, 97), poultry (Septicaemia, 24) and rabbits (GIT, 10). All strains were identified as *E. coli* on the basis of their cultural, morphological, biochemical and sugar fermentation reactions. Twenty two tests were performed (11, biochemical and 11, sugar fermentation reactions) to characterize and differentiating the strains from each other. One strain was found utilizing citrate as sole source of carbon and four strains were negative for indole test. More than 95% strains fermented lactose, arabinose, xylose, sorbitol and rhamnose while adonitol and inositol were fermented only by 5.6% and 1.0% strains respectively. Other sugars e.g. dulcitol, sucrose and salicin were fermented by 43% to 66% of the strains. On the basis of heterogenous fermentation of 5 selected sugars (i.e.. dulcitol, sucrose, salicin, raffinose and adonitol), 194 *E.coli* strains were grouped into 24 biotypes. The fermentation of dulcitol, sucrose and raffinose, were correlated for their association with virulence and pathogenicity of the *E. coli* strains, like pathogenic 'O' serogroups.

The serotyping of 194 strains of *E. coli* was carried out at National *Escherichia coli* Typing Centre Kasauli, (H.P.), India against available 'O' antisera. Out of 194 strains, 112 were typed and distributed among 54 'O' serogroups. Maximum number of 6 strains were recorded in two serogroups

(O35 and O9) followed by serogroups O2, O25 (5 strains each); O25, O60, O68, O103 and O147 (four strains each); O1, O61, O73 and O84 (3 strains each) O15, O17, O22, O23, O38, O45, O70, O88, O93, O98, O143, and O159 (two strains each) and rest 28 'O' serogroups had only one strain in each group.

Out of 17 known human pathogenic 'O' serogroups (as described by WHO, 1980), five (O20, O25, O44, O55 and O86) were encountered among test strains isolated from non-human primates. Similarly, known animal pathogenic O-serogroups (O5, O12, O106 and O143) were also recorded in human isolates in the present study.

These serological finding clearly, indicate free transmission of pathogenic *E. coli* strains from human to animals and vice versa, thus, such strains are of great importance in term of bacterial zoonoses.

In addition to the above known pathogenic groups of man and animals, five other human pathogenic O-serogroups (O1, O2, O15, O158 and O159) were also encountered in monkey diarrhoea which were generally not known to be pathogenic in non-human primates. Although all these strains were isolated from frank diseased conditions. Therefore, the present criteria based on biochemical and serological classification alone is not sufficient to differentiate a strain to be pathogenic or non-pathogenic. Thus, the emphasis was given on the presence of some other virulence factors among *E.coli* population and their association with each other.

PLASMID ENCODED DRUG RESISTANCE IN *ESCHERICHIA COLI* POPULATION :

Antibiotic sensitivity behaviour and their level of resistance (Minimum Inhibitory Concentration, MIC) have been determined against 11 drugs namely ampicillin (Ap), amoxycillin (Ax), tetracycline (Tc), doxycycline (Dx), streptomycin (Sm), chloramphenicol (Cm), Kanamycin (Km), co-trimoxazole (Co), nitrofurantoin (Fd), nalidixic acid (Nal) and norfloxacin (Nr). In this investigation, 56.7% strains were observed to be resistant to various drugs simultaneously in different combinations (ranging from one to eight drugs), out of which 69% strains were multi-resistant to three or more drugs. The over all incidence of drug-resistance was maximum for Tc (41.23%) followed by Dx, Sm, Ap, Cm, Ax, Co and least for Fd and Km (nearly 3.5% each). None of the strain was found resistant to nalidixic acid and norfloxacin which signify the clinical importance of these drugs in human and veterinary chemotherapy. Incidence of drug resistance was more (69%) among human strains as compared to animal isolates (54%).

Resistant strains showed a wide range of drug resistance level. MIC values ranged from 12.5 to 3200 ug/ml for various antibacterial drugs. High levels of drug resistance in a particular bacterial strain is an apparent indication of involvement of plasmid DNA, mediating such resistance.

All 111 drug resistant strains so obtained were tested for conjugal transfer of their R-plasmids. About 31.5% of resistant strains transferred their drug resistance either partially or completely to the recipient *E.coli* K-12 strain. A representative group of 35 drug resistant non-conjugable strains were further tested for mobilization of their drug resistance determinants with the help of mobilization factor X^+ of *E. coli* and a repre-

sentative group of 19 drug resistant (non-conjugable and non-mobilizable) strains were also studied for plasmid elimination assay using three curing agents namely norfloxacin, acridine orange and SDS. Seven strains mobilized their R-factors and 6 strains were made to cure their R factors by one or more of the curing agents. The data indicated that norfloxacin was better curing agent as compared to SDS and acridine orange for eliminating R-plasmids of *E. coli*. Thus, a total of 48 drug resistant strains were demonstrated to carry R-plasmids (transferable and non-transferable).

PLASMID ENCODED VIRULENCE FACTORS OF *ESCHERICHIA COLI*:

ENTEROTOXINS:

Enterotoxins of *E. coli* are supposed to be the cause of acute diarrhoea both in infants of human and animals. Out of 194 strains of *E. coli*, 99 (51%) producing enterotoxins. Majority (74%) of ETEC strains were ST produced while LT and ST/LT producers were encountered almost equal (13% each). The occurrence of enterotoxigenic strains among poultry was 52% and in human UTI, 48.48%. Occurrence of such toxigenic strains in UTI may have dangerous implications because by contaminating and colonizing the bowl, they will lead to diarrhoea.

An interesting observation was that the ST enterotoxins played major role in causing acute diarrhoea in animals including monkey as was evident by their high incidence in animal ETEC strains. Auto-transferable nature of enterotoxin plasmids (Ent-factors) was detected among 22.41% of 58 R^+ ETEC strains. R^-ETEC strains could not be studied for the nature

of transferability of their Ent-factor due to absence of any direct selection markers.

COLICINS:

Another important virulence factors of *E. coli* strains which are associated with intestinal and extra- intestinal infections is said to be colicin production. Some colicin plasmids (colicin V, B & M) harbouring strains are more pathogenic and virulent in nature. In the present study, colicin production was detected among 33 (17.52%) of the strains using *E. coli* B, *E. coli* Row and *E. coli* K-12 as colicin sensitive indicator strains. It was observed that *E. coli* Row was the most suitable strain for colicin detection. Inter-generic transfer of Col-plasmids to other pathogenic bacteria like *Salmonella typhimurium* was demonstrated. Unlike *E. coli* K-12, which is colicin sensitive, *Sal. typhimurium* was found to be resistant against colicins of all 33 strains. Therefore for colicin transfer studies *Sal. typhimurium* was used as recipient. It was also found that transferability of Col-plasmid was as common as transfer of R-plasmid.

HAEMOLYSINS:

To explore the probable role of haemolysins on the pathogenicity of *E. coli*, we tested all 194 strains on 6% defibrinated sheep blood agar plates. Out of these, only 27 (14%) strains produced haemolysins. Maximum occurrence (40%) of haemolytic strains was recorded among human UTI isolates, followed by GIT isolates of human (12.12%) and monkey (10.3%). Haemolysin transfer study showed that only 4 of 28 (27 + 1 standard = 28) haemolytic strains could transfer their HLY factors to one or other recipients (*E. coli* K12, *E. coli* PB-176 and *Salmonella typhimurium*).

In our efforts to cure the HLY-factors, we could get very limited

success. Out of 24 non-transferable haemolytic strains only 4 could be cured for their HLY-factors by treating with 3 curing agents (norfloxacin, acridine orange and SDS). Low percentage of transfer or curing of HLY-factors in majority of these strains may be due to the presence of haemolysin determinants on chromosome rather than on plasmid.

EFFECT OF HLY-FACTORS ON VIRULENCE AND PATHOGENI - CITY OF *E. COLI*:

To access the effect of haemolysin on pathogenicity and virulence of its host bacterium, 27 haemolytic *E. coli* strains were tested in Swiss mice. All strains were inoculated intraperitoneally (i/p) at the dose of $0.17x10^8$ C F U /mouse and observed for mice mortality patterns upto 7 days. Different strains gave different patterns of mortality in mice. On the basis of their mortality patterns, haemolytic strains were divided into three groups as virulent (70-100% mortality), partially virulent (69-20% mortality) and avirulent (< 20% mortality).

After transfer of haemolysin factors, 2 conjugants of K-12 were further tested for their virulence in mice along with non-haemolytic isogenic recipient parent (*E. coli* K-12) and their respective donor (Hly^+ strains). All haemolytic K-12 conjugants showed mortality in mice while non-haemolytic parent K-12 caused no mortality. The total mortality percentage of transconjugants (Hly^+) was however less than their donor strains. Moreover, four parent haemolytic *E. coli* strains (*E. coli* Hly^+) and after, their subsequent curing of HLY factors (*E. coli* Hly^-) were also examined similarly in mice models for any change in their toxicity behaviour. Non-haemolytic derivative of parent strains showed less mortality in mice than their homogenic haemolytic parent strains. These findings indicate that haemolytic factor to be pathogenic and enhances the virulence of its

host bacteria. However, HLY factor alone was not decisive for virulence but additive with other factors because some parent haemolytic strains were found to cause very low mortality or very less reduction in mortality after eliminating HLY- factors.

ADHERING FACTORS:

Bacterial adherence to epithelial cells by virtue of pili or fimbriae is a recognized virulence factor, which facilitates colonisation of the bacterium in the upper intestinal tract, urethra and urinary bladder etc. of human beings and animals. These plasmid mediated fimbriae also attach to erythrocytes causing observable haemagglutination. This agglutination of erythrocytes of various species by bacteria is an indirect evidence of adhering factors being present. Out of 194 test strains, 150 (77.32%) showed either mannose sensitive haemagglutination (MSHA) or mannose resistant haemagglutination (MRHA) or both, with the erythrocytes of human type A+, guinea pig, fowl, rabbit, sheep and bovine. The strains thus, were grouped into different haemagglutinating patterns depending upon the type of fimbriae or adhesins present on *E. coli* surface. The strains expressing mannose sensitive agglutination with guinea pig erythrocytes provided evidence for the presence of common type -1 pili which may have their role in colonization of urinary tract, vaginal mucosa, urinary bladder and the large bowl.

A total of 64 (42.6%) strains of *E. coli* tested positive for MRHA reactions with the erythrocytes of one or more species under study. There MRHA adhesins may have a crucial role in pathogenesis of *E. coli* infections. Human strains showing MRHA with human type A^+ blood provided evidence for the presence of colonization factor antigen -1 (CFA-I) while some of the human isolates showed MRHA with bovine and sheep eryth-

rocytes which may be due to the presence of CFA-II. The presence of various haemagglutination patterns (MSHA/MRHA) among animal isolates may be due to the presence of different plasmid encoded adhesins on bacterial surface. These adhesins could be transferred to non-adhesin bearing recipient strain by conjugation. We found that a considerable number of UTI strains, (23.8%) transferred their haemagglutinating character(s) to *E. coli* K-12 recipient.

CORRELATION STUDIES OF VARIOUS VIRULENCE FACTORS:

Strong relationship between enterotoxigenicity and adhering factors, was observed among ETEC strains. Nearly 79% of ETEC strains showed various haemagglutinating phenotypes as $MSHA^+$, $MRHA^+$ and $MSHA^+/MRHA^+$. Not only this a close correlation was observed between ST and LT/ST production with MRHA phenotypes which was detected in 31% and 33% of ETEC strains respectively whereas only 22% of LT producing *E. coli* strains showed MRHA reactions. Among human ETEC strains 35% could show MRHA reaction with human type A^+ erythrocytes which indicated the presence of CFA-I. Only two enterotoxigenic strains of human origin exhibited MRHA with sheep/bovine erythrocytes.

High affinity was observed between haemolysins and adhering factors. Nearly, 81.48% of the haemolytic strains were found to possess mannose resistant haemagglutinins. Correlation also existed between haemolysin production and enterotoxin production. About 41.4% of haemolytic strains of human UTI origin produced heat stable (ST) enterotoxin. Although, the role of enterotoxins in UTI cases has not been defined, it has been suggested that HLY-plasmids and ST plasmids might be having strong compatibility or affinity to recombine in *E. coli* isolates of UTI origin.

ASSOCIATION OF VIRULENCE FACTORS AND DRUG RESISTANCE :

In the present investigation enterotoxin production and drug- resistance was simultaneously recorded in 59% of ETEC strains, showing a close correlation between the two. This was found to be in higher in human ETEC strains than in animals ETEC strains. A total of 65% of R^+ ETEC strains were found multi-resistant to three or more drugs. Very high incidence (54.16%) of co-transfer of Ent-factors and R-factors was also observed. It showed that gene responsible for enterotoxins biosynthesis might be present on plasmids which have strong affinity to co-exist or to recombine with transferable R-plasmids. Such diarrhoeal isolates from monkeys, therefore, are of great zoonotic importance.

Further, production of colicin and its association with drug-resistance have been analysed. It was observed that like enterotoxins, colicins also have a strong affinity with drug resistance plasmids. About 57.57% of Col^+ strains were resistant to one or more of the drugs. Co-transfer of antibiotic resistance and colicin production was also demonstrated in at least four strains. The formation and spread of such genetic combination will be an adaptive advantage to the bacterium. They will successfully compete, survive, multiply and colonise in the gut and other tissues even in the presence of antibiotics.

In this study a high affinity was observed between drug - resistance and haemolysin production. Majority (62%) of the haemolytic strains were found resistant to one or more drugs at a time. Although, co-transfer of haemolysin factor and drug resistance was found in one strain. During

plasmid elimination studies, HLY-factors and drug resistance markers were eliminated simultaneously from four *E. coli* strains. These results showed that at least in above five haemolytic strains genetic markers for haemolysin and drug resistance are located on plasmids having strong affinity to co-exist in the host bacterium.

In the present investigation an appreciable percentage (68%) of *E. coli* strains having different patterns of haemagglutination (MSHA or MRHA) were also resistant to one or more antibacterial drugs. This showed that like all other virulence factors adhering factors also have strong correlation with drug resistance plasmids in *E. coli* strains.

The present study revealed that plasmids play a crucial role in the virulence and pathogenesis of *E. coli* strains and the that nature and outcome of an *E. coli* infection does not depend upon a single virulence factor alone but it is a multifactorial phenomenon. It has also been established that plasmid encoded virulence factors of *E. coli* strains have close affinity (compatibility) with drug resistance plasmids, which are widely present among Gram -ve bacteria and can easily be transferred to inter and intra-generic species. Therefore, occurrence of such genetic combinations (co-existence) of drug resistance and virulence factors in *E. coli* may be more frequent in future due to the powerful selection pressure created by extensive and indiscriminate use of antibiotics particularly, in developing countries like India. Besides, transferable nature of plasmids and wide spread occurrence of transposons both on bacterial plasmids and chromosome will further enhance the accumulation, formation and spread of multi-virulent plasmids, resulting in many fold increase in the virulence

of the host bacterium. Such plasmids will also have an extra advantage to the isolates involved in intestinal (diarrhoea) and extra intestinal infections of man and animals. Because of their free transmission from man to animals and vice versa, this will still create very serious zoonotic problem.

CERTIFICATE

This is to certify that work emboided in this thesis entitled **"Studies on Plasmid Encoded Virulence Factors in the Strains of *Escherichia coli* of Man and Animals : Compatibility with R-plasmids"** has been carried out by **Mr. Iqbal Ahmad** under our supervisions.

He has fulfilled the requirements for **Degree of Doctor of Philosophy in Agriculture (Microbiology)** of Aligarh Muslim University, Aligarh, regarding nature and period of investigational work. The work included in this thesis is original unless stated otherwise, and has not been submitted for any other degree.

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ACKNOWLEDGEMENT

My most humble thanks with gratitude are to the universal being ***The lords of all universe*** *who constantly inspires entire humanity towards knowledge, truth and eternal joys.*

It is my privilege to express my profound sense of gratitude to my esteemed teachers and research guides Dr. J.N.S. Yadava FNA, FNAAS, Emeritus Scientist, Division of Microbiology, Central Drug Research Institute, Lucknow (India) and Dr. Shamim Ahmad, Reader in Microbiology, Institute of Ophthalmology, J. N. Medical College, Aligarh Muslim University, Aligarh (India) for their proper guidance, fatherly behaviour, constructive criticism, unflagging interest and unflinching support. They introduced me to the fascinating realm of plasmid biology and nurtured in me the ability of independent work. I shall ever remain grateful to them for the initial push at infant stages of my research career.

I offer my deep regards to Dr. V.P. Kamboj, FNA, FNASc and Prof. B.N. Dhawan, FNA, FAMS, the present and the former Directors of Central Drug Research Institute, Lucknow and Dr. N.B. Singh, Assistant Director and Dr. G.P. Dutta, FNA, Scientist Emeritus, the present and former Heads, Division of Microbiology, C.D.R.I. Lucknow and Director, Institute of Ophthalmology, A.M.U. Aligarh, for their keen interest, necessary facilities provided during pursuit of this work.

I owe my sincere thanks to Prof. M. Shamim Jairajpuri, D.Sc. FNA, FNASc, FNAAS, Director, Institute of Agricultural Sciences, Aligarh Muslim University, Aligarh (India) and Prof. S.K. Saxena, Prof. Khalid Mahmood, (Late) Prof. Israr Ahmad, the former Coordinators, Agriculture Centre, A.M.U. Aligarh without whose help many hurdles would have become insurmountable.

I also express my deep sense of gratitude to all my teachers specially Prof. M.Saleemuddin, Prof. S.M. Hadi, and Prof. Nafees Ahmad of Aligarh Muslim University Aligarh and Dr. K.R. Bharadwaj, Director, National Animal Laboratory Centre, C.D.R.I., Lucknow for their valuable suggestions from time to time.

Delighted, I feel to convey my sincere thanks to Dr. S.K. Puri, Dr. H.B. Rajani, Dr. K.K. Kamboj, Dr. C.X. George, Dr. (Mrs.) M. Kazim and Dr. (Mrs) Renu Bajpai, C.D.R.I., Lucknow for their aesthetic suggestions and continued encouragements during the course of present investigation.

It is pleasure to express my thankfulness to my seniors and colleagues, especially Dr. Choudhary Mashkoor Ahamad, Dr. M.P. Singh. Dr. (Mrs) Kavita

Gupta, Dr. (Mrs.) Anju, Sangeeta, Ms. Aradhna, Kavita ,Shaheena and Nikhat for their constant cooperation during the course of this study.

It is unvanished truth that treatise would have never materialised in the absence of my friends with whom I shared my joys and sorrows. In naming them I am likely to miss many of them who contributed in different ways. Nevertheless, Drs. Ozair Aziz, Anser Azim, Faizan Khan, Reyaz Khan, Mohd. Ibrahim, M/S M.Alam Jaferi, A.B. Pant, M. Junaid, Navedul Haque, Mushahid Rizivi, N. Rehmani, Guru Dutt, Chauhan, need to be mentioned.

I also thank to (Mrs) Kanchan Duggal, Ms. Savitree, Mr. Josheph and M/s. Laser byte for immaculate typing and printing. Financial support in the form of research fellowships from University Grants Commission, New Delhi, India is thankfully acknowledged.

I feel immense pleasure to acknowledge with thanks the technical assistance, rendered especially by Shri Chandrika Prasad (Technical officer) Mrs. Nuzhat Kamal, Mr. M. Rizvi Mr. Ashok yadav (Lucknow) and Mr. Merajuddin (Aligarh) to accomplish this work.

My acknowledgment would remain incomplete without making a special mention for unstinted support, I received from my elder brothers Mr. Abu Sufiyan, Mr. Abu-Faiz, Mr. Ehasn Ahmad and Mr. Imran Ahmad. The tremendous cooperation, love and affection which I received from my mother, sisters, wife "SEEMA" and other family members is beyond my ability to give words.

Words go deep into sorrow when I recall my father who left me when I was studying in graduation course but these are his blessings which always remain the guiding light of my career.

Iqbal Ahmad

December 1993 ***(IQBAL AHMAD)***

ABBREVIATIONS

AO	Acridine Orange
Ap	Ampicillin
Ax	Amoxycillin
CFF	Cell free filtrate
Cm	Chloramphenicol
Col	Colicinogenic
Co	Cotrimoxazole
Dx	Doxycycline
EAEC	Enteroadherent *Escherichia coli*
EAggEC	Enteroaggregative *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
Ent	Enterotoxigenic
GILA	Guinea pig ileal loop assay
GIT	Gastroenteritis
HLy	Haemolytic
Km	Kanamycin
MRHA	Mannose resistant haemagglutination
MSHA	Mannose sensitive haemagglutination
Nal	Nalidixic acid
Nr	Norfloxacin
NSS	Normal saline solution
PBS	Phosphate buffer solution
R	Drug resistance
RILA	Rabbit ileal loop assay
SDS	Sodium dodecyl sulphate
Sm	Streptomycin
Tc	Tetracycline
TSB	Tryptic soy broth
UTI	Urinary tract infection

DEDICATED TO

" The work inscribed in these pages are dedicated to my beloved father late Mohd. Idris, whose vision, support and training helped me to attain the educational heights in the realm of modern day Science."

CONTENTS

INTRODUCTION

Diarrhoeal diseases are the third leading cause of morbidity and major cause of infant mortality in most of the developing and underdeveloped nations. In many rural areas nearly 25% of children die before they are five years old and in some areas, this figure may be as high as 35% or at time even 50% of all the children born. The average mortality of Asia, Africa and Latin America was estimated about 2 billion (WHO, 1980 and 1990). The toll will probably continue to be high if the immediate steps are not taken to prevent this scourage.

Development of newer diagnostic techniques led to the identification of new aetiological agents that were formerly not thought to be involved in causing acute diarrhoea. Among these the important ones are: *Vibrio cholerae* and other *Vibrio* sp., *Escherichia coli* , *Shigella* sp., *Salmonella* sp. *Campylobacter* sp., *Yersinia enterocolitica, Clostridium perfringens, Proteus* sp., *Staphylococcus* sp. Besides, some viruses viz., rotaviruses and protozoan parasites have also been implicated as the causative agents of diarrhoea.

Among these, *Escherichia coli* has emerged as a single most important pathogen causing diarrhoea world-wide. This organism causes approximately five episodes per child per year in the first two years of life, on an average 220 diarrhoeal episodes and 1-4 deaths per year in every 100 children in this age group (Merson *et al.*, 1978; WHO, 1980 and 1990). It has been estimated that only enterotoxigenic *E.coli* (ETEC) gives rise to 650 million cases of diarrhoea and 8,00,000 death annually in children under 5 year of age in developing countries (Black, 1986).

The impact of ETEC diarrhoea on animal health is also of very high magnitude. Animals are particularly susceptible to infections during the

two distinct periods - (i) 1-3 days post-partum (neonatal diarrhoea), (ii) during and after weaning period (post weaning diarrhoea). Reports appearing from all over the world indicate heavy economic losses due to morbidity and mortality in young calves, lambs and piglets because of diarrhoea alone. In USSR, there is an annual loss of 25.3% and 34.0% in new born calves and piglets respectively. Similarly, in the USA losses due to diarrhoea are estimated to be 48.6-71.2 million dollars annually (House, 1978). However, the estimations of the economic losses due to single disease agent are difficult and complicated due to a variety of factors, such as survey techniques, animal managerial schemes and geographic locations.

In India as well as in other developing or under-developed countries, where, there is no proper reporting system, this task is still more difficult. Moreover, considering the prevailing conditions of insanitation, poor hygienic conditions, malnutrition, over crowding and close association between man and animals, infections syndrome is expected to be of much higher magnitude.

Escherichia coli is also associated with many other disease conditions in adults such as: urinary- tract infections, septiceamia, metritis and mastitis in man or animals. In addition to these main diseases, *E.coli* is also producing colibacillosis (peritonitis, meningitis, enteritis, pyelitis, pyelonephritis, angiocholitis, salpingo-oophoritis, appendicitis, otitis, puerperalseptic) and oedema disease in pigs (Gyles 1986, Aksenova and Lisoskaya, 1987).

Diarrhoegenic categories of *E.coli* have been classified into 5 groups by Levine (1987) e.g. enterotoxigenic (ETEC), enteropathogenic (EPEC),

enterohaemorrhagic (EHEC), enteroinvasive (EIEC) and enteroadherent (EAEC). These *E.coli* strains produce one or more enterotoxins, cytotoxins, colonization factors and adhesins which might contribute to the development of diarrhoea, (Levine and Odelman, 1984; Mathewson et *al.* 1985; Levine 1987). Enterotoxigenic *E.coli* responsible for diarrhoea, liberates at least two types of enterotoxins: heat labile (LT) and heat stable (ST). In recent years, few more factors such as vero-toxins and cytonecrotic factors etc. have also been reported to be associated with the diarrhoeal agents. Hence, new findings and studies are adding more question to the disease complex rather than they have been answered.

In general, the virulence of a strain of a pathogenic species is determined by two factors: its invasiveness or ability to proliferate in the body of host and its toxigenicity or ability to produce toxin. But the researches in past decades about bacterial pathogenicity have been reached at this conclusion that some strains are non-invasive and non-enterotoxigenic, yet they are pathogenic and virulent. It means some other factors are also involved in bacterial pathogenicity.

Several structures and products of *E.coli* have either a demonstrable or a potential role in contributing virulence in the gut and other host tissues. These structures include flagella, capsule, cell wall components and pili (fimbriae). These special structures and products exhibited by bacteria e.g. the production of enterotoxins, colicins and aerobactins, haemolysins, adhering factors, and serum resistance have been documented to be closely associated with the pathogenic strains of *E.coli* which might contribute to the development of different pathological conditions.

Extensive use of antibiotics for the last 47 years as chemotherapeutic

agents for human and animal diseases, growth accelerators for animals, in cheese making, in canning industry as cold sterilizers, and in fish culturing, has considerably increased the drug resistant bacteria through out the world. Multiple drug resistant population has increased enormously which has created a serious chemotherapeutic problem for human and veterinary medicine.

Bacterial plasmids are extra-chromosomal, circular, double stranded DNA elements, that constitute a reasonably stable but dispensable gene pool. Each plasmid autonomously replicates and controls its own copy number. Under normal circumstances they are not essential for the metabolic machinery of the bacterial cell. Often, however, these genetic elements carry genes for supplementary activities such as resistance to various drugs, resistance against the lytic activity of serum, biosynthesis of toxins, colicins, haemolysins, adhesive antigens or for the degradation of various organic substances, to better survive in adverse environmental conditions or permit their host to compete successfully with microbes of the same or other species in the intestinal tract and other organs. Some plasmids are special in the sence that they have a gene encoding for their transfer from donor organism to recipient. Such plasmids are called as conjugable plasmids. Further, some of them have the capacity to mobilize the non-conjugable plasmids into the susceptible recipients. Such transfer can occur in many bacteria and almost in every genera of *Enterobacteriaceae*, and normal intestinal flora particularly in *E.coli*, which plays a major role as a reservoir for such plasmids. Whenever such strains happen to come in contact with their own pathogenic counterparts or other susceptible bacteria like *Shigella, Salmonella, Enterobacter* etc., they readily transfer their plasmid DNA to these pathogens making them resistant to various

drugs.

What makes the problem more serious, is the simultaneous transfer of drug resistance and virulence marker gene(s). Such strains are now being increasingly reported and it is very difficult to eradicate them. There is a real danger that R-plasmid bearing bacteria of animals with common pathogenicity for man and animals may infect man and there is the possibility that R-plasmids of animal origin may be transferred to human pathogens through normal commensal flora and vice-versa. Such plasmid populations in nature constitute an important problem of today's research, because they are of great concern to physicians, veterinarians and public health workers. Further, association of various virulence factors with each other and with drug resistance determinants needs exploration from two aspects (i) as to what extent such genes combination exist in pathogenic *E.coli* strains under natural conditions (ii) what is their additive or antagonistic effect on the virulence and pathogenicity of bacteria.

Unfortunately, less efforts have been made in this direction particularly among pathogenic *E.coli* strains of man and laboratory animals. The study on the above area of research will be of great veterinary and public health importance. It will also fill up a missing link in the ecology of *E.coli* infections and will also reflect the severity of zoonotic diseases. Study on such aspect of bacterial research will also signify the role of plasmids in bacterial pathogenicity and chemotherapy. The present investigation, thus, has been planed with the following aims and objectives :

(1) To isolate, identify and characterise large number of *E.coli* strains from different diseased conditions of human and animals and to assess them for their antibiotic-resistance status.

(2) To study the extra-chromosomal nature of drug-resistance by *in vitro* conjugation, mobilisation and curing experiments.

(3) To screen all *E.coli* strains for their plasmid encoded virulence factors like enterotoxins, colicins, haemolysins and adhering factors.

(4) To evaluate the individual virulence factor for its pathogenicity by transferring it to a non-plasmid bearing recipient strain or by eliminating (curing) such factors from parent bacterial hosts.

(5) To see the co-existence (compatibility) of various genetic determinants coding for virulence factors with drug-resistance markers.

REVIEW OF LITERATURE

ESCHERICHIA COLI ASSOCIATED WITH VARIOUS DISEASED CONDITIONS OF MAN AND ANIMALS:

Escherichia coli occupies an important position because on one side it is supposed to be a normal inhabitant in the human and animal intestine and on the other side it needs vigilance as a pathogen. It is one of the most important organism in gastroenteritis and urinary tract infections. The role of *Escherichia coli* in causing outbreaks of infantile diarrhoea under 5 year of age with heavy morbidity and mortality among children have been well recognized. *E. coli* infections have also been reported among farm and laboratory animals and their zoonotic nature is documented (Sojka, 1965; WHO, 1967, Bharadwaj and Thomas 1974; Yadava *et al.* , 1984). Linton (1977) have described possible mode of transmission of *E. coli* from man to animal and vice-versa (Fig-1). Very little information are available on the monkey diarrhoea due to *Escherichia coli* . Ewing et *al.* (1955), Groven *et al.* (1969) and Needam (1975) have reported outbreaks of diarrhoea among laboratory monkeys. In female monkeys diarrhoea occurred in two phases one with in 10 days of delivery and second, after seven weeks.

Heavy morbidity and mortality rate among piglets of the age group since birth to 12 weeks, due to *E. coli* diarrhoea have been reported by many workers (Chen *et al.*, 1984; Djonne, 1985; Pohl *et al.*, 1988). Strains isolated belonged to 'O' serogroups 8, 48, 138, 141, 147, 149 and 157. *E. coli* strains have also been implicated as cause of tympanitis and diarrhoea in rabbits (Cantey and Hosterman, 1979, Moon *et al.*, 1983; Camgulhene and Milon 1989) Strains of O103 serogroups have been found most prevalent among diarrhoeal isolates of *E. coli* from weaned rabbits.

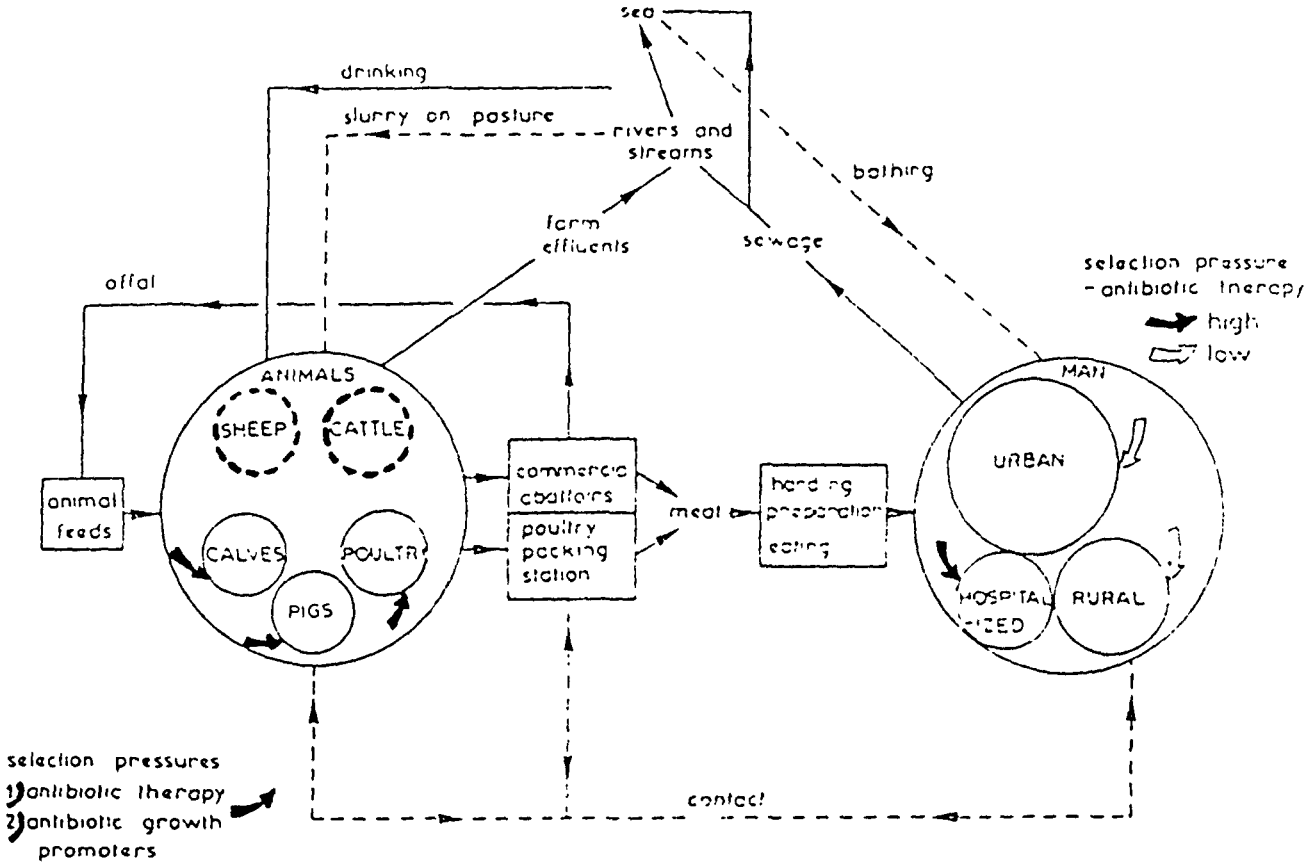


Figure 1: Zoonosis: *Escherichia coli* flow diagram between farm animals and poultry on the one hand and the human population of the other as shown by Linton (1977).

Ugo-Chukawu and Anukam (1988) have reported *Escherichia coli* as the major cause of diarrhoea among African dwarf goats.

Diarrhoea due to *Escherichia coli* occurs most frequently in calves less than 1 week of age but can also be a problem in calves as old as 2-3 weeks or also in elder calves (Acres *et al.*, 1977; Yadava *et al.*, 1984). Heavy economic losses due to heavy morbidity and mortality of calves due to *E. coli* diarrhoea have been documented (Farid *et al.*, 1976; Jank *et al.*, 1990). Bovine diarrhoegenic strains of *E. coli* mainly belongs to a number of O:K serotypes (O8:K25, O8: K85, O9:K30, O9:K35, O101:K28 and O101:K30). A dysentery syndrome in calves have been associated with non-ETEC strains that adhere to enterocytes and cause damage to micro villi as well as cause a mild/acute inflammation of the underlying lamina as described by Gyles (1986).

Diarrhoegenic *E. coli* strains have been classified into five categories by Levine (1987). His classification is mainly based on distinct virulence properties, different interactions with intestinal mucosa, distinct clinical syndromes, differences in epidemiology and distinct O:K:H serotypes. These categories are as follows :

i) ENTEROTOXIGENIC *E. COLI* (ETEC):

ETEC produced enterotoxins heat stable (ST), heat labile (LT) and both (ST/LT) are important cause of diarrhoea in infants, young children, adults and neonatal calves as well as in adult animals. ETEC strains tend to fall with in restricted O:H serotypes. A correlation between serotypes and enterotoxin types exist for example, O6:H16 usually produced LT/ST while O128:H12 almost always produce ST, likewise O27: H7, O27:H20, O128:H12, O153:H10 are mainly ST producers. (Orskov and Orskov, 1978

and Reis *et al.* , 1979)

ii) ENTEROINVASIVE *E. COLI* (EIEC).

Like *Shigella*, enteroinvasive strains of *E. coli* invade and proliferate within the epithelial cells and cause damage and death of the cells. The invasive nature of EIEC group is dependent on the presence of plasmid (Harris *et al.*, 1982). *E. coli* of this group mainly falls in 'O' serogroups : O28, O29, O124, O136, O143, O144, O152, O164, O167. EIEC cause sporadic food born infection and outbreaks of diarrhoea (*Shigella* like dysentery) in all ages, world wide (WHO, 1990).

iii) ENTEROPATHOGENIC *E.COLI* (EPEC):

In some urban area upto 30% of acute diarrhoea cases in young infants are attributed to EPEC. The mechanism of EPEC diarrhoea is not yet clear. EPEC mainly belongs to certain restricted serotypes such as O18, O20, O26, O44, O55, O86, O111, O112, O114, O125, O127, O128, and O142 (Orskov and Orskov 1978; Levine, 1987). Adherence property of EPEC (Localised or diffused adherence to Hep-2 cells) are mediated by plasmids (Nataro, *et al.*, 1985 and Fletcher *et al.*, 1992).

iv) ENTEROADHERENT *E.COLI* (EAEC):

E. coli of this group neither produced LT, ST, or Shiga-like toxin nor invades epithelial cells. The mechanism involved in the pathogenesis and sero-groups of this category are yet to be established. Preliminary works suggest that the *E. coli* of this group are identifiable by a particular pattern of adherence to Hep-2 cells which is clearly distinguishable from both localized as well as diffused adherence (Mathewson *et al.*, 1986). EAEC causes watery diarrhoea in young children which may become persistent (WHO, 1990).

v) ENTEROHAEMORRHAGIC *E.COLI* (EHEC) :

EHEC causes sporadic haemorrhagic colitis in North America and some other countries. Occasionally, direct person to person transmission may occur. EHEC produces a cytotoxin which may be responsible for oedema and diffuse bleeding in the colon resulting in bloody diarrhoea as well as the haemolytic - uraemic syndrome, that some times develops in children. *E. coli* serogroups O157 has emerged as the single most important enteric pathogen causing bloody diarrhoea (Karmali *et al.*, 1985; WHO, 1990; Scotland *et al.*, 1990).

Recently a putative category has been described as enteroadherent-aggregative *E. coli* (EAggEC) identifiable by the characteristic aggregative pattern of adherence of bacteria in Hep-2 cell assay (Nataro *et al.* , 1987 and 1992). Bhan *et al.* (1989) referred this newly characterised group as enteroaggregative *E. coli* (EAggEC) and they found that EAggEC are typically negative with DNA probes that identify other categories of diarrhoegenic *E. coli* and do not fall into any specific O:H serotypes, typical for other categories of diarrhoegenic *E. coli*. In addition to intestine *E. coli* is the most common pathogen in urinary tract infection (UTI) in human and can also be a problem in animals like cats, dogs and buffaloes (Osborne and Klausmer, 1979; Hacker and Hughes, 1985; E.L. Rauf et al., 1985 and Gyles, 1986).

Beside causing diarrhoea *E. coli* has also been known to cause a variety of pathological conditions in man and animals. The organism is associated with infections of urogenital-tract, peritonitis, hepatitis, gall-bladder infections, pneumonia and meningitis (Charter, 1956; Reis, 1958; Omprakash, 1962; Bhat *et al.*, 1964). In cattle it is known to cause naval infections, joint ill, pylonephritis, cervicitis, metritis, mastitis, abortions,

colibacillosis and oedema disease in pigs (Gracey, 1955; Anon, 1959; Sojka, 1965). In poultry it is known to cause colisepticaemia, coligranuloma, omphalitis, amphaloflabitis, peritonitis and chronic respiratory disease (Sojka, 1965; Sojka and Carnagham 1961; Prasad *et al.*, 1967; Yadava and Malik, 1971). In small laboratory animals like rats, mice, guinea pigs and rabbits it is known to cause septicaemia with suppurative abscesses.

E. coli associated with different disease conditions have several potential virulence properties and these properties may directly contribute to the pathogenic potential have been shown to be plasmid specified. Plasmids that code for several distinct toxins, haemolysins, colicins, fimbrial adhesins as well as certain surface antigens, were frequently associated with pathogenic *E. coli* isolates.

DRUG-RESISTANCE STATUS OF ESCHERICHIA COLI STRAINS :

Until recently, the great medical importance of plasmid has been the most antibiotic resistance seen in the hospital is mediated by plasmids. Now it has become apparent that these plasmids (R-plasmid) also specify properties that directly or indirectly contribute to pathogenicity and virulence of bacteria. Therefore, it becomes necessary that drug resistance plasmids of pathogenic *E. coli* isolates should be dealt with accordingly.

Extensive and indiscriminate use of antibiotics for the treatment of various clinical infections in man and animals, and as an additives used in animal feed causes in the emergence of resistant strains throughout world. Multiple drug resistant population has increased enormously which has created great problem in human and veterinary chemotherapy.

Although new antibacterial agents have been developed and search for newer ones is in progress to combat with resistant pathogens, the bacteria find new ways to circumvent the new drug as well. Davies and Smith (1978) have suggested variety of mechanisms which can be responsible for such increased resistance in bacteria : **(i)** Alteration of the target site in the cell that reduce or eliminate the binding of the drug to the target site. **(ii)** Blocking the transport of the antibiotic into the cell regardless of whether or not specific or active mechanisms of drug transport are involved, because a change in the transport system can reduce the penetration of the drug into the cell. **(iii)** Increasing the level of enzyme inactivating the drug, so that drug is "Saturated" and titrated out. **(iv)** Decreasing the cells metabolic requirement for the pathway or reaction inhibited by the drug. **(v)** Providing the cell with replacement for the metabolic step that is inhibited by antimicrobial agent (by pass mechanism). **(vi)** Detoxification or inactivation of the antibiotic with increased production of enzymes. **(vii)** Production of a metabolite that can antagonise the inhibitory effect of the inhibitor.

Considering these possible mechanisms of resistance we can say that mechanisms **(i)**, **(ii)**, **(iii)** and **(iv)** could easily be attained by point mutations in chromosomal structure or by regular genes in the absence of any such events. However, for many antibiotics, it is unlikely that mechanisms **(iv)**, **(v)**, or **(vi)** could arise by simple mutation of chromosomal genes. In these cases entirely new cellular functions are required that could be provided by the inheritance of new genes in the form of plasmids i.e. positive function resistance.

Kitamoto and co-workers (1956) were the first to discover transferable drug resistance in 1955 in *Shigella* isolated from human dysentery

cases in Japan. It carried drug resistance factors against sulphonamide (Su), streptomycin (Sm), chloramphenicol (Cm) and tetracycline (Tc). In U.K. it was first demonstrated by Datta (1962) and found the resistance against kamamycin (Km) and neomycin (Nm) in addition to the above four antibiotics.

In India, the first survey of this type was conducted by Yadava in 1962 at Central Drug Research Institute, Lucknow. He reported (1966) multiple drug resistance (14%) among *E. coli* strains against six antibiotics namely penicillin (Pn), streptomycin (Sm), tetracycline (Tc), chlortetracycline (CTc), oxytetracycline (OTc) and chloramphenicol (Cm) simultaneously. These strains of *E. coli* were isolated from buffalo calf diarrhoea and were found to be highly resistant to as many as 6 antibiotics (Pn, Sm, OTc, CTc and Cm) simultaneously. These animals neither received any antibiotics in their feed as additives or as growth stimulants, nor were given any antibiotic therapy before the isolation of these strains.

R-factors have been postulated by Watanabe in 1963 to consist of a linear linkages of two genetic determinants (i) Drug resistance determinants and (ii) Resistance transfer factor (RTF). Anderson (1965) demonstrated that RTF and R-determinants behave as basically independent genetic units which have affinity to associate with each other to form R-factors, when they happen to be present in the same cell (Fig. 2). Occurrence of transposons both on bacterial chromosome and on plasmids has greatly influenced the spread of drug resistance marker gene (Hedges and Jacob, 1974; Rilay and Anilionis, 1978; Nuquent *et al.*, 1979; Datta, 1980; Threlfall *et al.*, 1983).

Trishkina *et al.* (1977) determined sensitivity of 300 *E. coli* strains

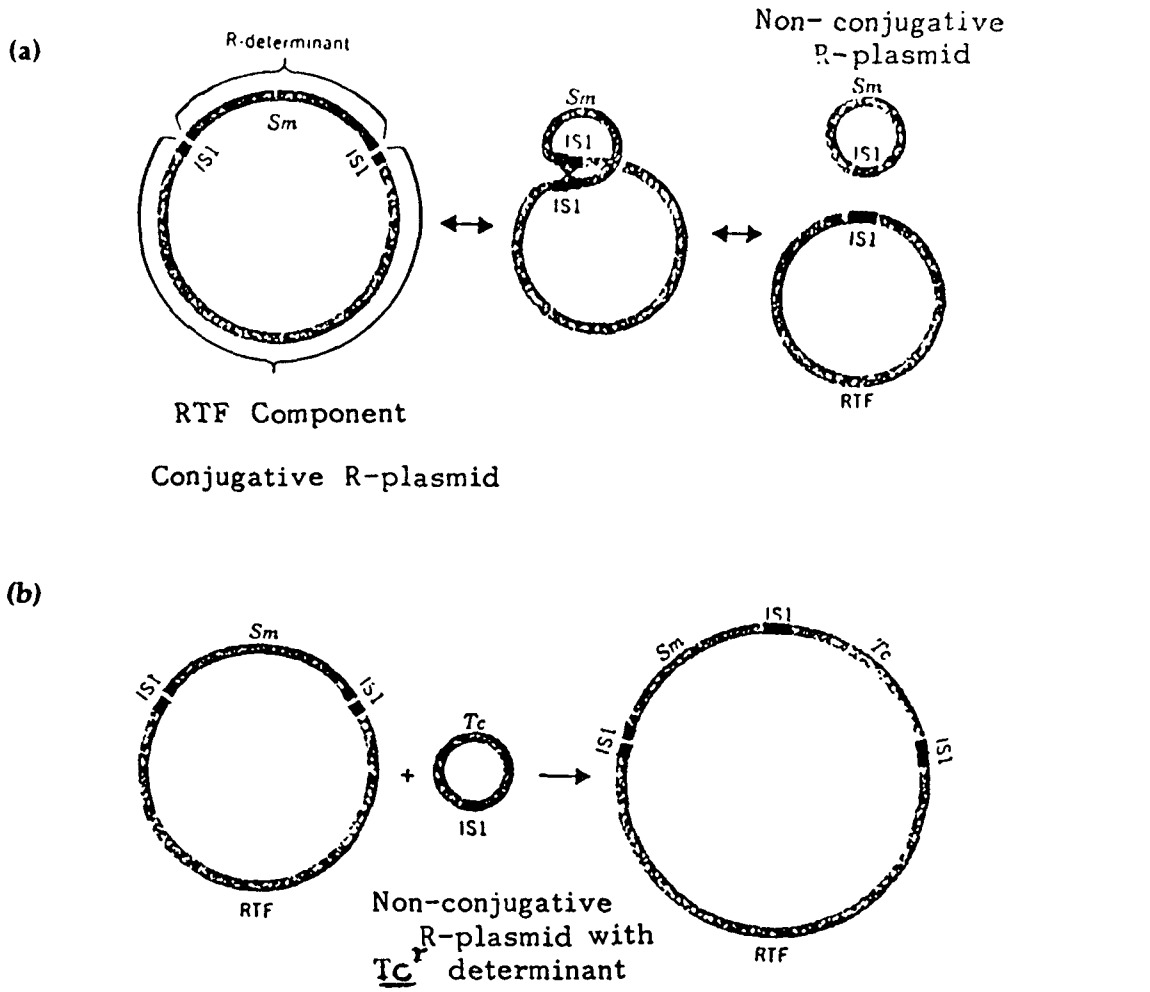


Figure 2 : **(a)** Structure of conjugative and non-conjugative plasmids.

(b) Proposed mechanism for the evolution of compound R-plasmids.

(mainly O141, O35) against seven antibiotics isolated from calves. About 75 to 90% of the strains were resistant, of which 64% were multiple drug resistant. Treatment with polymyxin alone or with furazolidone was found most effective.

Kariuki (1977) reported high incidence of multiple antibiotic resistance in *E. coli* strains isolated from diarrhoeal calves. Mostly the strains were resistant to Su, Tc and Sm. None of his strain was found resistant to furazolidone or Neomycin. Out of 193 strains, 105 (54.5%) could transfer their resistance inter-generic to *Salmonella typhimurium.*

Diwan and Sharma (1978) studied 200 strains of *E. coli* from UTI and reported 87.5% of the strains to be resistant to one or more antibiotics. They observed maximum resistance against Tc (90.2%); Ap (81%) and Cm (78.2%). Nearly 55% of the resistant strains were able to transfer their resistance partially or completely to the recipient *E. coli* K12 by conjugation. Nakamura *et al.* (1978) reported the drug resistance pattern and R-plasmid distribution in faecal *E. coli* isolates, 68.8% of the bovine and 89.9% of the porcine isolates were found resistant to one or more of the antibiotics (Tc, Cm, Sm, Km, Pm, Na) Transferable R-plasmids were carried by 57.7% of the bovine and 52.2% of the porcine resistant isolates.

Sarkar *et al.* (1979) reported multiple drug resistance in 71% of the strains from 250 enteropathogenic *E. coli* strains, isolated from diarrhoeal diseases in children. The most common pattern of resistance were Ap, Cm, Km, Nm, Sm; Ap, Cm, Sm and Ap, Sm. None of the strains was resistant to gentamicin and only nine were resistant to furazolidone. Adetosye (1980) studied drug resistance and distribution of R-factors among 333 *E. coli* isolates from clinically healthy and sick animals. Fifteen multiple and

three single antibiotic resistance pattern were found. Out of 285 resistant strains, 133 (39.7%) transferred their resistance determinants to sensitive *E. coli* K12 recipient.

Al-Sowaygh and Shibl (1981) studied 1818 isolates of enterobacteria from faecal specimens and reported 50.2% isolates to be resistant to one or more of the 10 antibiotics they tested. About 0.8% of *E. coli* isolates were found simultaneously resistant to 5 antibiotics (Am, Cm, Sm, Tc and Carbenicillin), but none of them was resistant to gentamicin. Incidence of self transferable R-plasmid was recorded 40% among 86 resistant strains tested. Agarwal *et al.* (1981) demonstrated that 81.5% of the resistant strains possessed transferable drug resistance factor. Ranganekar *et al.* (1982) studied 181 *E. coli* strains, isolated from healthy and hospitalised patients and found 79% strains resistant to one or more drugs. They reported that 84.6% of the resistant strains carried R-factors.

In 1983 Bridge and Manning observed 60% of transferable R-plasmid among 100 multiple resistant strains of *E. coli* . Agarwal *et al* . (1984) studied 285 *E. coli* strains of human origin for their antibiotic sensitivity behaviour. Multiple drug resistance was observed in 76.5% of the strains. They observed 42.6% of their strains to have transferable plasmids. Wise *et al.* (1985) reported trimethoprim (Tp) resistance plasmid among 1572 strains of *E. coli* isolated from the cases of diarrhoea in cattle, pigs and sheep. Resistant to trimethoprim was detected 28% among of bovine isolates, 13% of porcine isolates and 9% of ovine isolates. Nearly 45% of resistant isolates transferred their Tp resistance to *E. coli* K12 and 17% strains were shown to carry non-self transferable plasmids which were capable of being mobilized to *E. coli* K12 by Rp_4 plasmid.

In a study of 284 strains of Enterobacteria from Vellore, Young *et al.* (1986) administrated 64% of the strains resistant to trimthoprim. They also observed 60% strains to have transferable plasmids in *E. coli* and identified 58 different plasmid types. Helin and Araj (1986) studied 1253 isolates of UTI from children and adults for their sensitivity against 13 drugs. They observed a high resistance among *E. coli* strains against Ap, Su and cotrimoxazole.

Infectious nature of R-plasmids coding for aminoglycoside resistance among Gram -ve bacteria has been widely studied. (John and Twitty, 1986; John and Mc-Neil 1981). Singh and John (1991) have reported the presence of multiple plasmids DNA band > 50 Kb in size (Inc N- group) among six tobramycin resistant *E. coli* strains.

The ß-lactam antibiotics have been used more extensively than any other group of antibiotics for treatment of clinical infections and such wide usage of these agents had led to considerable selective pressure for the development of bacterial resistance. The rapid increase of ß-lactam resistance in recent years has become major problem specially among Gram-negative bacteria causing various infections. Incidence of ß-lactam resistance among enterobacteriaceae specially in *E. coli* have been reported world wide. (Roy *et al.*, 1983; Simpson *et al.*, 1986; Reid et *al.*, 1988; Medeiros *et al.*, 1989).

Various types of ß lactamases (plasmid/chromosome mediated) which inactivate ß-lactam antibiotics is the major resistance mechanism found in bacteria. (Sykes *et al.*, 1981; Bush, 1988; Thomson *et al.*, 1990; Payne and Amyes, 1991 and Amyes and Gemmell (1992). Singh *et al.*, (1992) have reported higher level of resistance (MIC, 12 to 3200 mg/L)

among resistant strains of *E. coli* against ampicillin, tetracycline and cotrimoxazole. Cross resistance and lack of cross resistance among ß-lactam antibiotics (ampicillin, amoxycillin and cloxacillin) were reported. The inability of cross resistance among ß-Lactam antibiotics is due to the different substrate specificity of different ß-lactamases (Bush, 1988).

In the last few years many workers have studied the role of extended spectrum ß-lactamases in the development of drug resistance against ß-lactam. Extended spectrum of ß-lactamases are plasmid mediated enzymes that confers resistance to oxyimino ß-lactam such as cefotaxime, ceftazidine and other broad spectrum cephalosporins and to monobactams such as aztreonam (Paul *et al.*, 1989; Jacoby and Sutton, 1991; Jacoby and Medeiros, 1991).

Nalidixic acid, the first quinolone, was introduced into clinical practice in the early 1960s. The 6-fluorinated quinolones which are significantly superior, in particular in intrinsic activity and antibacterial spectrum, have been increasingly used since the 1970s. 4- quinolones are chemically synthesised compounds whose principle target is the enzyme, DNA gyrase (E.C. number 5.99.1.3)., the only bacterial enzyme capable of introducing negative supercoils into DNA (Suttcliffe *et al.*, 1989). In the 1980s compounds such as pefloxacin, norfloxacin, ciprofloxacin, enoxacin, and ofloxacin, active against a wide range of bacterial species became available for clinical use.

Plasmid mediated resistance to 4- quinolones, nitrofurans, novobiocin, polypeptides, and rifampicin are as yet to be identified in clinical isolates (Courvalin, 1990). Chromosomal mediated resistance to the 4-quinolones can occur either an alteration in the target enzyme DNA gyrase or a

mutation that reduces drug accumulation. Mutation in the subunit of DNA gyrase coded by the gyr-B gene have been shown to cause 4- quinolone resistance in *E. coli* and *Ps. aeruginosa* (Nakamura *et al* , 1989, Yoshida *et al.*, 1990). However unlike gyrase-A mutation these do not always cause cross resistance to all 4- quinolone permeability. In Gram -ve bacteria mutations affecting 4-quinolones have been identified in *E. coli* (nal-B, nfx-B, nor-B, cfx-B) and other bacteria (Piddock and Wise, 1989; Lewin *et al.* , 1990).

ELIMINATION OF PLASMIDS FROM BACTERIAL CELLS BY CERTAIN CHEMICALS:

Elimination of plasmid mediated drug resistance is of practical significance both in chemotherapy of bacterial infections and in microbial genetics. The first critical study of plasmid elimination by chemical (acriflavin) was conducted by Rownd *et al.* (1966). Although Hirota and Iijima (1957) and Hirota (1960) used acriflavin and acridine orange and were able to produce F^-cells of *E. coli* from F^+ cells.

Plasmid elimination is not only useful to detect the plasmid mediated characters but also to produce plasmid free derivatives. Such derivative of strains are very useful in studying the role of particular plasmid encoded factors in virulence and pathogenicity of bacterium. Keeping in view the plasmid DNA replication mechanism, various approaches to eliminate the plasmid DNA have been made, such as : **(i)** Direct inhibition of DNA synthesis by intercalating dyes. **(ii)** Inhibition of DNA replication by alkylating agents or inhibition of synthesis of functional proteins. **(iii)** Dissolution of cell surface by surface acting agents. **(iv)** Elevation of temperature (May *et al.*, 1974; Stalder and Adelberg, 1972). **(v)** Nutritional

starvation of plasmids bearing bacteria (Pinney and Smith, 1971; Bremer *et al.*, 1973; Yadav *et al.*, 1984). **(vi)** Ultra violet irradiation (Rownd *et al.*, 1966; Wamburker and Panse, 1982).**(vii)** Incompatibility grouping (Toh-E and Wickner, 1981). **(viii)** Protoplast formation and regeneration (Novic *et al.*, 1980).

None of the above mentioned approaches for elimination of plasmid DNA, is able to cure all plasmids of all groups. However, DNA intercalating agents, surface acting agents and recently introduced compounds of 4 quinolone group (antagonizing A-subunit of DNA gyrase) are supposed to be the agents of choice to eliminate *E. coli* plasmids.

An anionic surface acting agent, sodium dodecyl sulphate (SDS) has been reported to cure R and F factors in *E. coli* with very high efficiency (Tomoeda *et al.*, 1968; Salisbury *et al.*, 1972; Kaur *et al.*, (1985); Singh and Yadava, 1988).

Various DNA inter-calating compounds have been reported to eliminate the plasmid selectively without affecting the chromosomal replication system such as acridine orange (Watanabe and Fukasawa, 1961 b; Mitsahashi *et al.*, 1961; Singh and Yadava, 1988); ethidium bromide (Bouanchaud *et al.*, 1969).

Inhibition of plasmid DNA replication has also been reported by various other compounds that either inhibit protein synthesis or inactivate the functional proteins particularly at various stages of transcription and translation or block the process of replication which ultimately lead to the loss of extra-chromosomal DNA in subsequent progenies. Such compounds are rifampicin (Bazzicalupo and Tocchini-Valentini, 1972; Obaseki-Ebor, 1984) which binds to the B-subunit of RNA polymerase and inacti-

vate it, thus stopping the transcription; Mitomycin C (Iyer and Dzybalski, 1964) which inserts occasional alkylating cross links between two strands of double halical DNA. This cross linkage of DNA blocks transcription by DNA and prevents replication.

Among antibacterial drugs inhibiting DNA replication by acting on A or B subunit of DNA gyrase have been reported to have potential to cure plasmids. The bacterial DNA gyrase is a tetramer consisting of two A and two B subunits (Suttcliffe *et al.*, 1989). Hooper *et al.*, (1984) have reported the elimination of plasmids PMG 110 from *E. coli* by novobiocin and other inhibitor of DNA gyrase.

PLASMID ENCODED VIRULENCE FACTORS OF *ESCHERICHIA COLI* :

ENTEROTOXINS :

The role of enterotoxins in causing diarrhoea by enterotoxigenic *E. coli* (ETEC) in man and animals has been proved by many workers (Larsen, 1976; Smith and Huggins, 1978; Harnet and Gyles, 1983; Klipstein *et al.*, 1983; Saxena and Yadava, 1985; Levine, 1987). Today enterotoxins produced by ETEC has been recognized as one of the major etiological agent for causing acute diarrhoeal diseases (Echeverria *et al.*, 1984; WHO, 1980 and 1990).

The discovery of rabbit ileal loop model (De and Chatterjee, 1953), led De and his colleague in 1956 to show that whole broth cultures of several *E. coli* including enteropathogenic *E. coli* serotypes from diarrhoea, resulted in fluid secretory response in rabbit ileal loop assay (RILA)

which did not occur with non-toxigenic strains kept as control. However they could not implicate toxin as the cause of secretory response. The preliminary experiments conducted by De (1959) and De *et al.* (1960) clearly demonstrated the presence of some toxic material in the cell free culture filtrate of *Vibrio cholerea* (Cholera toxin). This filtrate was capable of causing dilation and increasing fluid accumulation in the ligated ileal segments of rabbit. These basic experiments led to the discovery of enterotoxins in the cell free filtrate of *E. coli* as also described by Gyles and Barnum (1967), Smith and Halls (1967,a,b) and Glew *et al.*, (1968). Enterotoxins as described by Craig (1972) are the agents responsible for increased movement of water and electrolytes from plasma compartment to Lumen of small intestine. Thermolabile eneterotoxin (LT) was detected by Smith and Halls (1967 a) among *E. coli* cell free filtrate. Its property of toxicity was lost when it was subjected to heat treatment at 65°C for 15 minutes. Kohlar (1968) found simultaneously another type of toxin from *E. coli* strain, whose activity was not lost by heating the cells free culture filtrate at 65°C for 15 minutes and thus this toxin was named as thermostable enterotoxin (ST) of *E. coli* . An enterotoxigenic *E. coli* can produce both or either of the two types of enterotoxins: Heat labile and/ or heat stable (Smith and Gyles, 1970; Gyles, 1971).

Biochemical nature of heat labile enterotoxins has been extensively studied during past two decades for full evaluation of its role in diarrhoea. Gyles and Barnum (1969) indicated that LT enterotoxin is immunogenic and antigenically related to cholera toxin. The molecular weight of LT toxins ranged between 20,000 to 102,000 daltons (Evans *et al.*, 1978). CT and the related *E. coli* LT are heterohexameric proteins produced in the intestinal lumen by respective bacteria during infection (Jacob *et al.*, 1984)

and composed of a central A-subunit surrounded by 5-B subunits with molecular weight of 11,780 daltons each (Dallas *et al.*, 1979). A subunit is estimated as 25,000 dalton to 30,000 dalton and contains two fragments A1 and A2 of mol. wt. 21,000-23,500 and 4000-6000 respectively, linked by a disulphide bond (Kunkel and Robertson, 1979).

The amino acids composition of B subunit of both LT and CT shows similarities, while the A subunits are different (Clements *et al.*, 1980). The nucleotide sequence of these two toxins are approximately 80% homologous (Dallas and Falkow, 1980). Subseqent studies on LT enterotoxins elaborated its biochemical and molecular structure to a great extent (Evans *et al.* , 1974; Clements and Finkelstein, 1978; Kazemi and Finkelstein, 1990, Gupta and Yadava, 1992). Recently the crystal structure of toxin exhibiting an unusual AB5 architecture of molecule have been reported (Sixma *et al.*, 1991 and 1992), as depicted in Figure 3.

The role of LT toxin in the pathogenesis of diarrhoea have been well studied. Both A and B subunits of LT are synthesized intracellularly as precursor proteins. After translocation across the bacterial cytoplasmic membrane and removal of leader peptides, the AB5 complex is assembled in the periplasm. After release from bacterial cell the LT toxin binds to intestinal epithelial cells (using GM1 gangliosides) of the host by B-subunit LT toxin activate cyclic AMP system and causes sharp increase in cAMP concentration. Ultimately leads to the development of watery diarrhoea. (Moss and Vaughan, 1988; Field *et al.*, 1989; Spangler, 1992).

Heat stable enterotoxins (STs), produced by enterotoxigenic strains of *E. coli* is low molecular weight of 1000 and 10,000 daltons, poor or non antigenic, dialysable peptide and capable of causing 50% to 80% of re-

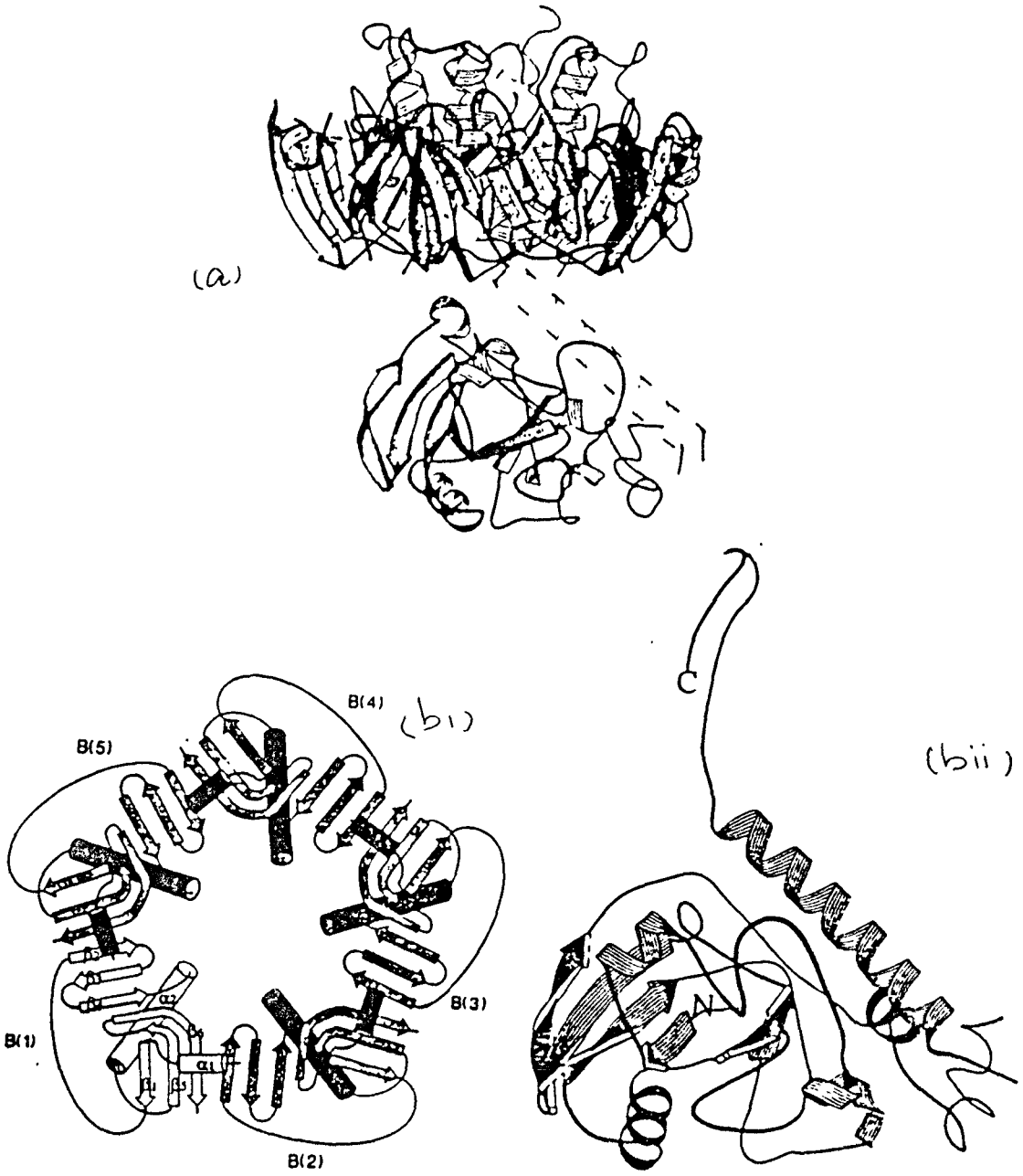


Figure 3 : (a) Ribbon plot showing the AB5 complex of LT enterotoxin, Note the free end of small helix and tail of the C terminus of the A1 sub unit (centre, base of pentamer), Sixma *et al.* (1991).

(bi) Schematic secondary structure diagram. (projection of the B pentamer. A single monomer is depicted in light colour, (Sixma *et al.*, 1991)

(bii) Ribbon diagram of the A subunit showing the A1 subunit folds and the long helix of A2, terminated bya small helix and tail. (Sixma *et al.*, 1991)

ported cases of diarrhoea in developing countries (Giannella, 1981) and also a major cause of diarrhoea in laboratory and domestic animals (Burgess *et al* , 1978). There are two major subsets of ST group of toxins. STa or ST-1 and STb or ST-II. STa is characterized by methanol solubility and its ability to induce accumulation of fluid in the intestine of suckling mice that have received toxin orally or intragastrically (Gyles, 1979; Greenburg and Guerrant, 1981).

Saxena and Yadava (1982) have developed a very sensitive animal model (Guinea pig ileal loop assay) for the detection of ST enterotoxin of *E. coli* . Choudhary *et al.* (1991) further evaluated this system and found a better replacement with infant mouse model because this system was found sensitive to both types of ST toxins (STa and STb).

The toxin (STa) contains 18 or 19 aminoacids, with the active site in the terminal 14 aminoacids portion. The structure is characterized by the presence of six cysteine residue which involved in disulphide linkage (Klipstein 1983; Gyles 1986 and 1992). The STa toxin binds to a cell surface receptor (not well defined) in the intestine, which subsequently leads to an activation of guanylyl cyclase and then stimulate fluid secretion by rise in cGMP concentration and effecting Ca channels. (Field *et al.*, 1978; Guerrant *et al.*, 1980; Huott *et al.*, 1988). ST-b gene code for a 71 aminoacid peptide containing a single peptide of 23 amino acids and a mature protein of 48 aminoacids (Lawrence *et al.*, 1990; Debreuil *et al.*, 1991). The mechanism of action of ST-b toxins is not understood. However, Hitotsubashi *et al* (1992) have pointed out that prostaglandin E 2 might be playing an important role in the mechanism of action of ST-b toxins.

Genetics of *E.coli* enterotoxins : Genetic determinants responsible for toxins production are located on auto-transferable or non-transferable plasmid designated as "Ent-plasmids". (Gyles *et al.*, 1974). Exceptional cases of location of gene for LT in chromosome (Green *et al.*, 1983) and in bacteriophage (Takeda and Murphy, 1978) have also been reported. Ent-plasmids are transferable in nature by conjugation to *Salmonella typhimurium, S. dublin, Shigella flexneri, Proteus vulgaris, Klebsiella aerogenes, Pseudomonas aeuroginosa* and *E. coli* strains. (Smith and Halls 1968; Yadava, *et al.*, 1986). Nucleotide sequence encoding for the production of STa enterotoxin have also been found to be located on a transposon (So *et al.*, 1979) flanked by ISI elements. Gene for STb was also found to be located in DNA segment flanked by the inverted repeats. (Mazaitis *et al.*, 1981). Some Ent-plasmids carry genes for LT, some for ST and others for LT/ST both. Gyles *et al.* (1974) demonstrated auto-transferable nature of Ent-plasmid in 17% toxigenic *E. coli* isolates and also determined the molecular size of heat liable and heat-stable enterotoxin encoding plasmids, to be 6.0×10^7 and 2.1×10^7 daltons respectively. Ent-plasmids range in size from 20-150 Kilobase but most of them are in 80-100 kb range. Recently, Lortie *et al.* (1991) have reported that out of 4, ETEC-strains from human stool, 2 strains have the genes coding for heat-stable enterotoxins (ST-b) as detected by colony hybridization assay. The ST-b gene was found to be the located on a 70 Kb plasmid which was also coding for heat-labile enterotoxin (pLT-1).

Compatibility of Ent-plasmids with drug resistance :

Although, only the enterotoxin and colonization antigen plasmids have been shown to have a direct role in pathogenicity. ETEC strains routinely harbour five or more distinct plasmid species. One or more of

these plasmids is frequently an antibiotic resistance plasmids. The antibiotic resistance determinants, toxin genes, and adherence gene are usually found on separate plasmid species. However, several plasmids encoding both toxin production and antibiotic resistance have also been reported.

Wachsmuth *et al.* (1976) found that 36% of a multi-resistant *E. coli* population produced ST toxin and that all toxigenic strains were resistant to antibiotics. The plasmids responsible for enterotoxin production (ST) and multiple drug resistance was found to have MW $67x10^6$ and $30x10^5$ dalton respectively. Gyles *et al.* (1977) found 90% of the enterotoxigenic strains of piglet diarrhoea to be resistant to one or more antibacterial drugs. They further observed that 50% of these strains transferred their Ent-factor alongwith drug resistance markers to *E. coli* K-12 strain. In another study Gyles *et al.* (1978) reported en-bloc transfer of Ent and R-factors in the intestine of newly weaned pigs.

Echeverria and Murphy (1978) have described a plasmid, about 180 megadaltons in size coding for the specific heat-labile enterotoxin synthesis as well as resistance to Tc and Sm. This plasmid was thought to be the result of recombination between a 60 megadalton R-factor and an Ent-plasmid in a strain isolated from a case of human infantile diarrhoea. Co-existence of drug resistance gene(s) and genetic determinant(s) coding for biosynthesis of enterotoxins on the same plasmid have been documented among *E. coli* strains (Scotland, 1979; McConnell *et al.*, 1980; Stieglitiz, 1980; Silva, 1983; Danbara *et al.*, 1988, Singh *et al.*, 1992)

Saxena and Yadava (1985) have shown co-transfer of antibiotic resistance and enterotoxigenicity by 39% of the *E. coli* strains. They

Ap determinants than Cm and Tc. Thomas *et al.* (1987) have also confirmed that the expression of ST, drug resistance and surface-antigens CS-5 and CS-6 were mediated by plasmids and could transferred simultaneously. Choudhary *et al.* (1988) have reported prevalence of drug resistance and enterotoxins production among 149 strains of *E. coli* of farm animals. They observed high incidence of multiple drug resistance among ETEC, strains of farm animal origin and showed that LT enterotoxin production was more closely associated with drug resistance than ST and LT/ST production. Co-transfer of Ent-factor and drug resistance were observed in four strains.

Danbara *et al.* (1988) observed simultaneous transfer of ampicillin resistance and enterotoxin gene in 20.1% ETEC strains suggesting that these strains contain conjugative plasmids. Lamikanra *et al.* (1990) reported that out of 70 strains of *E. coli* they studied, 32.9% were resistance to multiple drugs and 54.3% of the strains harboured transferable R-plasmid and 40% of ETEC strains could transfer trimethoprim resistance with LT toxin gene.

Singh *et al.* (1992) reported a close association among 13 enterotoxigenic *E. coli* with antibiotic resistance markers. Out of these anterotoxigenic strains, nine were found harbouring transferable R-plasmids. Only one strain of serogroup O32, transferred ampicillin, oxytetracycline and doxycycline resistance and heat labile enterotoxin biosynthesis determinant en-bloc. Plasmid DNA analysis of the exconjugants showed the presence of a 41.8 MDa conjugative plasmid.

COLICINS AND COL-PLASMIDS:

Colicins are antibiotic like substances that are produced by certain members of the family Enterobacteriaceae especially by *Escherichia coli* .

These are highly specific and act upon strains of the same or closely related species (Fredriq-1957). Other bacteria were later found, however, that produce similarly acting substances, requiring the broader term "bacteriocins". Over 20 distinct type of colicins have been described, among which colicin, V, A, B, Ia, Lb, K, N and E are well documented (Pattus *et al.*, 1990). These colicins although differ in their precise modes of killing, share a number of features in common. Bacteria are immune to the specific colicin they carry. Colicin is generally released following cell lysis or quasi-lysis. There are usually three colicin related genes, colicin, lysis and immunity genes. (Nomura, 1967; Pugsley, 1984). Like F- factors, colicin factors were described before it was recognised that these factors were encoded by plasmids (Fredriq, 1957). These plasmids are either small, high copy number plasmids or large, conjugative plasmids (Pugsley, 1987).

Most characterized colicins range in molecular mass from 27,000 to 80,000 Dalton (Hardy, 1975; Konisky, 1982). Colicin V on the other hand is a small molecule (Hershman *et al.* , 1967). Further more colicin V is not released from bacterial cell wall by means of cell lysis (the typical mechanism for most colicins) but rather appears to be exported (Gilson *et al.*, 1987). The pore forming colicins like A,B,EI,Ia, Ib and K induced increased permeability by ion channel formation (Pattus, 1990) but the colicin V does not do so. The specific target of colicin V is thought to be the inner cytoplasmic membrane, where the membrane potential is disrupted by an undefined mechanism (Yang and Konisky, 1984; Water and Crosa, 1991).

The role of colicins for the survival and establishment of bacteria in competition with other non-colicin producing intestinal bacteria gives extra advantage to colicin producer which enhance the virulence of bacterium. (Yadava and Gupta, 1971; Smith, 1974; Smith and Huggins, 1976,

Ansari and Yadava, 1984; Chao and Levine, 1981).

Smith and Huggins (1976) and Goel *et al.* (1980) found that colicin plasmids bearing *E. coli* strains were more pathogenic and they survive and multiply better in alimentary tract of experimental animals as compared to homogenic non-colicinogenic strains. The strains bearing Col-V plasmid, when injected intramuscularly in chickens were found more pathogenic as compared to homogenic non-Col-V strains.

All of Col-V plasmid by definition encode colicin-V but may or may not encode other properties related to bacterial virulence. The other properties that have been frequently associated with colicin V-plasmids includes, colicin V-plasmid transfer related functions, aerobactin iron uptake system, increased serum survival, resistance to phagocytosis, change in motility and hydrophobicity with intestinal epithelial cell adherence (Water and Crosa, 1991). Since Col-plasmids are in most cases transferable and could spread from an antibiotic sensitive strain to a resistant strain *in-vivo*, providing "quantum leaps" toward virulence (Falkow *et al.*, 1987).

There have been several reports on high incidence of colicinogenic *E. coli* strains from Northern part of India (Yadava and Gupta, 1971; Ansari and Yadava, 1981; 1984 and Singh *et al.* , 1989). Arunachalam *et al.* (1974) isolated 59 strains associated with colisepticaemia and enteritis in poultry and found that 22 (35%) of their *E. coli* strains were colicinogenic. Schal (1975) studied 103 *E. coli* strains, isolated from piglets and calves died for colibacillosis, of these 45% were colicinogenic. One the other hand 29% colicinogenic strains were detected by them among 352 strains isolated from faecal samples of healthy calves also. Njoku-Obi *et al.* (1978) studies 419 *E. coli* strains recovered from human pathological materials in

Nigeria, 51/ 555(9%) strains were found to be colicinogenic. Colicinogenic type B was the most frequent among strains of faeces while type-A predominated among the strains from UTI, followed by equal distribution of B_1, E_2 and I_a colicins. Binns *et al.* (1979) found that elimination of Col V factor from human, bovine, ovine and avian strains invariably reduced their pathogenicity for experimental animals.

Ansari and Yadava (1981) studied 310 strains of *E. coli* of human and animal origin for the production of colicins. They reported 13.5% strains as colicinogenic. Higher incidence of colicinogeney was reported in animal strains as compared to strains from human origin. Surprisingly 55% of the buffalo strains were recorded as colicinogenic followed by strains from cows (24.6%), mares (4.4) and poultry (3.6%). Aguero *et al.* (1983) reported increase Col-V plasmids and manose -resistant haemagglutination (MRHA) in an *E. coli* K1 population and they discussed that presence of these properties may be playing a role in the ability of some *E. coli* K1 serogroup to invade the host tissue. Yadava *et al.* (1986) have reported 9.2% of *E. coli* strains of human origin were colicinogenic and 64% of Col^+ strains were resistant to one or more drugs. Similar study was also conducted by Singh *et al.* (1989) among domestic animal isolate and reported co-transfer of Col-factor and R-factors among 2 colicinogenic strains to *E. coli* K12.

In the recent years incidence of colicin production among clinical isolates of *E. coli* of man and animal origin and their extrachromosomal nature (Col-Plasmid) have been reported by many workers (Blanco *et al.*, 1990, Bradley, 1991, Riley and Gordon 1992). Fernandez-Beras *et al.* (1990) have pointed out that the colicin-V genotype was predominantly chromosomal in diarrhoeal Isolates and predominantly plasmid encoded in bacteremic

isolates. This has been considered as the circumstantial evidence for the greater importance of colicin-V in the gut.

HAEMOLYSINS:

Kayser (1903) reported that cultures of *E. coli* and their supernatants lysed erythrocytes. Dudgeon and Pulvertaft in 1927 also reported the haemolytic activity in *E. coli* but could not demonstrate haemolysin in culture filtrates. Some other workers also reported the existence of cell bound haemolytic activity of *E. coli* strains (Bamforth and Dudgeon, 1952). Smith (1963) employed erythrocytic agar medium for the recognition of haemolytic *E. coli* in faecal sample of different man and animals. He reported a number of haemolytic *E. coli* strains from faecal sample of cattle (67%), pigs (63%), sheep (53%) and man (18%) in different out breaks of gastro-enteritis. He also differentiated clearly, the cell bound (ß-haemolysin) and cell free (α-haemolysin). α-haemolysin antiserum neutralized α-haemolysin but could not inactivate ß-haemolysin, whereas antiserum made against ß-haemolysin had no neutralizing activity. These findings indicated that these 2 haemolysins might be different (Smith, 1963; Short and Kurtz, 1971; Rennie and Arbuthnott, 1974)

A second type of filterable cell free haemolysin (organic acid) was detected when an haemolysin producing *E. coli* strain was grown in a chemically defined medium (Jorgensen *et al.*, 1975). A third type of haemolysin called θ- haemolysin was detected in *E. coli* strains which were resistant to nalidixic acid. The haemolysin does not lyse RBCs of human and rabbit but lyse RBCs of other species (Walton and Smith, 1969).

Genetics of haemolysins : The transmissible nature of the haemolytic character of *E. coli* was first described by Smith and Halls (1967) they reported that haemolytic phenotype assumed to be encoded by a plasmid

designated as "Hly". Noegel *et al.* (1981) reported that α - haemolytic *E. coli* strain-MM 152 harbour 3 transmissible plasmids which could be transferred to recipient strain *E. coli* K-12. With isolated plasmids they could show that the genetic determinant required for haemolysin was located entirely on plasmid. Their data suggests that there are at least three clustered cistron which are required for haemolysin production.

Extra-chromosomal nature Hly-encoding genes were also studied by curing Hly factor from bacterial strains. Mitchell and Kenworthy (1977) found that Hly factor could be eliminated at high frequency by RNA inhibitors such as (actinomycin-D, rifampicin and streovaricin) as compared to intercalating DNA inhibitors on plasmid replication (like acriflavin, ethidium bromide, daunorubicin and ethyl violet) which are less effective. The different Hly plasmids were found to be heterogenous in size, incompatibility groups and conjugational behaviour, but all share large genetic homology in their α-haemolysin determinants (Welch and Falkow, 1984; Hacker and Hughes, 1984;). Genetic location of Hly-determinants on bacterial chromosome rather than on plasmids, have been reported by many workers (Minshew *et al.* , 1978; Hull *et al.* , 1982; Colonna *et al.* , 1992; Falbo *et al.* , 1992).

Despite the minor structural differences, the cloned chromosomal α-Hly determinants of serologically different *E. coli* strains share over all genetic homology (Berger *et al.*, 1982). This was also found when plasmid and chromosomal Hly determinants were compared (Muller *et al.* , 1983; Knapp *et al.*, 1984; Hacker and Hughes, 1985).

Spontaneous loss of Hly-determinants flanked by insertion sequences have been reported in-vivo and in-vitro by bacteria (Low *et al.*, 1984; Beutin *et al.* , 1986; Hacker *et al.* , 1983, 1990). On the basis of above

finding and other known literature various workers suggested that Hly-determinants have spread by transposition like events (Zabala *et al.*, 1982; Hacker and Hughes, 1985; Knapp *et al.*, 1985; Koronakis *et al.*, 1987). Little is know about the genetics of other *E. coli* haemolysin. In contrast to α-haemolysin, the production of enterohaemolysin is determined by temperate bacteriophages in EPEC serotypes 026 strains. Enterohaemolysin and α-haemolysin do not share DNA homology (Beutin *et al.*, 1988 and 1989).

Haemolysins in relation to virulence of *E. coli* :

Haemolytic activity is attributed for increased bacterial virulence and pathogenicity of bacterium. (Sojka, 1965; Cabello, 1979; Ahmad and Yadava, 1980; Evans *et al.*, 1981; Hull *et al.*, 1982; Cavalieri *et al.*, 1984; Alonso *et al.*, 1987).

Epidemiological studies have shown that haemolysin production correlates with *E. coli* causing UTI and sepsis. An association of α-haemolysin with virulence of some *E. coli* was detected in experimental infections of laboratory animals (Minshew *et al.*, 1978; Hacker *et al.*, 1983; Hughes *et al.*, 1983). Quantitative difference in virulence correlate with the amount of haemolysin produced in isogenic *E. coli* carrying different recombinant α-Hly plasmids (Welch *et al.*, 1981; Welch and Falkow, 1984; Hacker and Hughes, 1985).

Smith (1963) showed that crude culture supernatants of α-haemolysin are toxic and lethal for mice. Emody *et al.* (1980) could not demonstrate substantial differences in toxicity between α-haemolysin producing culture and their non-haemolytic derivatives by intravenous injection of mice. However, when introduced intra-peritonealy or intranasally, haemolytic organism were clearly more toxic (Ketyi *et al.*, 1978; Smith and Huggins,

1985). However the observation that the increase in bacterial *E. coli* host back ground indicate that additional factors play a role in animal toxicity (Waalwijk *et al.*, 1982; Hacker *et al.*, 1983).

Berger *et al.* (1982) as well as Muller *et al.* (1983) reported that the structural gene (Hly-A) for haemolysin from different isolates of haemolytic *E. coli* show some variation in nucleotide sequence. This suggests that difference in virulence of *E. coli* isolates or in toxicity of a haemolysin molecule might be a reflection of amino acid composition. Whether, there are different toxicities associated with different haemolysin preparations is unknown or whether quantitative difference in haemolysin production can count for the differences is still unclear.

A partially purified preparations of α– haemolysin from human isolates are cytotoxic for human leucocytes (Cavalieri and Synder 1982; Vagts *et al.* 1993) and for fibroblast *in vitro* (Cavalieri and Synder 1982 b), lysis of RBC's may result in making iron available for growth of bacterium (Linggood and Ingram, 1982 Waalwijk *et al.*, 1983). The finding that α-haemolysin interferes with phagocytosis and has a toxic effect on phagocytes indicate that it play an important role in *E. coli* infections by counteracting a major host defence mechanism (Gadeberg and Larsen 1988; Bhakdi *et al.*, 1989; Gadeberg *et al.*, 1989). In experimental models the haemolysin elicit pulmonary hypertenstion and vascular leakage in isolated rabbit lungs and may, thus contribute to acute respiratory failure in septicemia (Grimminger *et al.*, 1990).

The role of α-haemolysin in enteric infections is less clear. However,α haemolysin producing *E. coli* have a clear selective advantage when compared with nonhaemolytic strains. Besides it appears possible that

haemolysin contribute as a virulence factor in enteric infections of new-born infants by P-fimbriated *E. coli* (Wold *et al.*, 1988; Gillard *et al.*, 1989; Beutin *et al.*, 1990 b).

The α-haemolysin is frequently associated with certain pathogenic factors such as production of P-fimbriae, certain capsular antigens, serum resistance, high toxicity for mice and mannose resistant haemagglutination (Hughes *et al.*, 1983; DeBoy *et al.*, 1983; Czirok *et al.*, 1986; Low *et al.*, 1984; High *et al.*, 1988; Zingler *et al.*, 1990). None of these factors absolutely correlated with virulence, rather it appears that pathogenicity of a strain is determined by a combination of a number of contributing factors.

Hacker (1989) and Hacker *et al.* (1990) presented data on the genetic linkage of a Hly determinant and the gene encoding P-related fimbrial adhesins in isolates belonging to sero group O6 and O4. They also showed that virulence gene block may be part of larger DNA region which may be deleted from the chromosome and proposed for these regions the term, "Pathogenicity DNA island".

Fule *et al.* (1990) reported prevalence of haemolysin, haemagglutination and antibiotic resistance among 56 urinary isolates of *Escherichia coli*. They could not found correlation among these virulence factors with drug resistance determinants.

Prada *et al* (1991) reported that α-haemolysin was closely associated with the production of cytotoxic necrotising factor (CNF). It was also found that all strains carrying ST plasmids were negative for CNF in canine *E. coli* isolates. CNF production seems to be closely associated with the production of chromosomally encoded haemolysin, where as plasmid

production of chromosomally encoded haemolysin, where as plasmid coded Hly are more often associated with ST production. Such strains are negative for CNF production. Recently, Hariharan *et al.* (1992) have reported a strong association between production of LT toxin and haemolysin among 52 *E. coli* strains of pig diarrhoea. Ninety percent of 29 Hly^+ strains were LT^+ whereas 100% of 23 Hly^- isolates were LT^-.

ADHERING FACTORS ASSOCIATED WITH PATHOGENIC *ESCHERICHIA COLI* STRAINS :

The ability of certain bacteria to adhere to eucaryotic cells have been recognised as a fundamental feature for the colonization of host tissue in-vivo. For many pathogenic bacteria including *E. coli.* adherence is of paramount importance since they have to compete with the commensal micro organism for a successful colonization of the host epithelial cell. The adhesive property of bacteria to intestinal cell receptors was first discovered by Guyot (1908) who observed that some strains possessed the ability to agglutinate RBC's from a number of animal species. Although similar observation were made by subsequent investigators. (Rosenthal, 1943; Kauffmann, 1948). The most common terminologies for these structure responsible for adhesive property of bacteria are "fimbriae", introduced by Duguid *et al.* (1955) and `pili' introduced by Brinton (1965). Many authors (Ottow, 1975; Jones, 1977; Gaastra and de-Graaf, 1982) used the term fimbriae, which are thinner, more numerous than flagella, proteinaceous, filamentous appendages on the bacterial cell surface.

These structure are capable of provoking immunological reactions and also used for serological classification of pathogenic and non-patho-

genic strains (Gaastra and deGraaf, 1982). The most common antigens of *E. coli* are the O, K and H antigens. The terminology O (from the German : Ohne Hauch) and H (from the German : Hauch) introduced by Weil and Felix (1918) is used for somatic (O) and flagellar (H) antigens of *E. coli* . The term K antigens (from the German word Kapsel) as introduced by Kauffman and Vahlne as described by Gaastra and deGraaf 1982 . The O antigen, also called as somatic antigen, which are not inactivated by heat at 100°C or 121°C for one and half hr. It is lipopolysaccharide moiety of the bacterial cell wall.

The H-antigens are the protein in nature, thermolabile and are inactivated by heat at 100°C. The K-antigens are usually acidic polysaccharide which form envelop or capsule around the cell wall. The K antigen can be sub-divided into three categories L, A and B, where L antigens are thermolabile, A is thermostable and B is thermo labile. So far 164 types of O antigens, 103 K antigens, and 75 H antigens have been recognized (Ananthnarayan and Paniker, 1989). A new type K antigen designated as K88 from oedema disease and enteritis in swine was described by Orskov *et al.* , (1961). It was further recognized that certain serotypes of *E. coli* associated with porcine neonatal diarrhoea have K88 antigen on the surface (Sojka, 1973). Smith and Linggood (1972) reported an antigen from enteropathogenic *E. coli* isolated from calves and lambs, designating as Common K antigens or 'KCO' which was later named as K99 (Orsko *et al.*, 1975). The K88, K89 and F41 adhesins were associated with animal isolates of ETEC strains and were found pathogenic for cattle, swine and Lambs, whereas K88 expressing strains were primarily pathogenic for swine as described by Moon (1990) and Korth *et al.* (1992).

Enterotoxigenic *E. coli* strains of the porcine origin lacking K88 antigen were found to possess another adhesin designated as 987P. These fimbrial adhesins were found to be encoded by plasmid/chromosome (Gasstra and de Graaf, 1982; Casey *et al.*, 1990). Evens *et al.* (1975) described a surface antigen with similar characteristics to that of K88 antigens on ETEC of human origin (Strain H-10407). This antigen was termed as colonization factor antigen (CFA). Two other immunologically distinct surface antigens from human ETEC were recognized and named as CFA/I and CFA/II (Evans and Evans 1978).

CFA-I have a single homogenous fimbrial antigens, CFA-II, consist of three sub components called coli surface antigen (CS1, CS2 and CS3) and CFA-IV have also an antigen complex comprising CS4, CS5 and CS6. (Evans and Evans, 1978; Cravioto *et al.*, 1982; Levine *et al.*, 1984). Honda *et al.* (1984) have described another colonization factor as CFA III. All above colonization factors associated with human ETEC strains described above requires plasmid gene for expression and these are usually associated with enterotoxin coding gene as described by Hibbered *et al.* (1991).

Various fimbrial adhesins of *E. coli* isolates frequently agglutinate a wide range of man and animals (guinea pig, fowl, sheep, bovine, horse, pig and monkey) RBC's as described by Duguid and Old (1980). Haemagglutination may be mannose sensitive (MS) or mannose resistant (MR), depanding upon the sensitivity to D-mannose (Evans *et al.*, 1979; Gaastra and deGraaf, 1982; Clegg and Gerlach, 1987; Eisentien, 1988).

The majority of both pathogenic and non-pathogenic *E. coli* strains produce type-1 fimbriae which gave MSHA reaction with erythrocytes of several animal species. (Salit and Gotschlich, 1977). The significant role

of type-1 fimbriae in *E. coli* colonization of the urinary tract, vaginal and bladder, mucosal surface and large bowl and their role in pathogenicity of EPEC strain have been reported by several workers (Ottow, 1975, Brinton, 1967; Reid and Sobel 1987; Finlay and Falkow, 1989).

Among *E. coli* strains in particular of diarrhoeal and extra-intestinal infections cases a number of distinct fimbrial antigens have been identified which gave MRHA with erythrocytes of limited number of animal species (Oudega and Graaf, 1988; Moon, 1990). Mannose resistant (MR) fimbriae were found to be clearly associated with virulence of the bacteria of diarrhoeal and extra intestinal infection cases (Orskov and Orskov, 1985; Saxena and Yadava, 1985; Blanco *et al.*, 1990 and 1991; Nihal and Turet, 1992). The occurrence of serotypes from diarrhoeal out breaks gives evidence of the association of O:H serotypes with enterotoxin production various O sero-groups and O:H serotypes associated with diarrhoegenic isolates of *E. coli* (Table 1).

Evans *et al.* (1977) showed that K88, K99 and CFA (I and II) demonstrate specificity as haemagglutinins. Stirm *et al.* (1967) showed that K88 antigen of porcine ETEC has ability to agglutinate with guinea pig erythrocytes in the presence mannose, while K99 antigen exhibits mannose resistant haemagglutination with horse or sheep erythrocytes. Evans *et al.* (1977) showed that mannose-resistant haemagglutination of human types A erythrocyte give evidence of the presence of CFA/ I. Later on Evans and Evans (1978) identified CFA/II as new surface associated heat-labile colonization factor antigen, which could haemagglutinate with erythrocytes of bovine and chicken in presence of mannose.

Fimbrial adhesins of extra-intestinal origin, expressing MRHA are

Table 1 : Some of the 'O' serogroups and O:H serotypes associated with diarrhoegenic strains of Escherichia coli

Source	Serotypes	References
Children and adults .diarrohoea (mostly ETEC)	06:H16, 08:H9, O15:H11, O25:H42, O78:H11, O78:H12, O8:K40:H9, O149:H10	Oroskov, et al. (1976 and 1977)
Human diarrohoea	O8:K40:H9, O6:K15:H16 O111:H1, O111:H2, O119:H6	Goldhar, et al. (1980) Toledo, et al. (1983)
Infantile diarrhoea	O18, O20, O25, O26, O28, O44, O55, O86, O111, O112, O114, O119, O125, O126	WHO, (1980)
	O63, O77, O139, O159, O167	Blanco, et al. (1991)
	O127, O128, O142, O158	Levine, (1987); Devine, (1989)
New born piglets diarrhoea	O8 :K87, O45,O138: K81, O141: K85, O147:K89, O149:K91, O157	Sojka, (1971)
Calves diarrhoea	O25, O55, O86, O112, O119, O125, O126	Yadava and Gupta, (1969)
Bovine diarrhoea	O8, O138, O147, O149	Wray, et al. (1993)

P, S, M and X fimbriae (Vaisanen *et al.*, 1981). P-fimbriae which represent the main group of mannose resistant fimbriae associated with urinary tract infection posses adhesins, which recognise the globo-series of glycolipids present on the human erythrocytes possessing the P-blood group antigen (Vaisanen -Rhen *et al* , 1984; Stromberg *et al.*, 1991). S-fimbriae adhesins recognize α–sialyl-(2-3) ß-Gal-containing receptor structure and are commonly found on *E. coli* strains causing sepsis in new born meningitis (Hacker *et al.*, 1985).

It was found that 10-50% of uropathogenic *E. coli* recognize receptors other than diagalactoside and mannose binding specificities and these have been referred as to X adhesins. Girardeau *et al.* (1988) also showed variable haemagglutinins among *E. coli* strains from different clinical conditions. These adhesive antigen as expressed by MRHA of human, chicken and sheep erythrocytes are encoded by plasmids as demonstrated by conjugative experiments (Pal and Ghose, 1990).

Various workers have shown correlation between enterotoxin production and presence of adhesins. According to Gyles and Barnum (1969) and Gyles *et al.* (1974). The K88 antigen was mostly found in association with strains producing heat labile and heat stable enterotoxins. Moon *et al.* (1976) and Isaacson *et al.* (1978) have shown that K99 antigen has been associated with the ST production. Smith *et al.* (1979) demonstrated the presence of a single non-conjugative plasmid of $60x10^6$ daltons carrying genes for CFA/I and ST enterotoxin. Penaranda *et al.* (1983) transferred ST:LT:CFA/II, genes in *E. coli* K-12 which were located on a single plasmid of about $60x10^6$ daltons. A close association exists between the presence of CFA-/I and the production of ST+LT or ST only (Evans *et al.*, 1977 and 1978; Smith *et al.*, 1979; Levine *et al.*, 1980; Lopez-Vidal *et al.*, 1990).

An interesting observation was made by Casey *et al.* (1992) which showed that two ETEC strains of pig diarrhoea origin could not express K88, K99, F41 or 987P adhesins and do not cross react the antisera raised against these adhesins but were causing diarrhoea in pigs. Their study concluded that these pathogenic *E. coli* strains expressed a common surface antigen that may be novel adhesins in *E. coli* strains.

MATERIALS AND METHODS

BACTERIAL STRAINS :

Escherichia coli strains were isolated from following sources of man and laboratory animals. Broadly, these strains can be divided under following headings.

Human Isolates:

i) Faecal samples of patients showing symptoms of gastroenteritis both male and female of different age groups admitted in Infectious Disease Hospital (Balrampur), Lucknow, and sporadic diarrhoeal cases of animal laboratory attendants of Central Drug Research Institute, Lucknow, were collected for isolation of *Escherichia coli* strains.

ii) Urine samples were examined from outdoor and indoor patients suffering from urinary or uro-genital tract infection at above hospital, Lucknow.

Animal Isolates:

i) Non-human Primates (Monkeys): The Rhesus, (*Macaca mulata*), Bonnet (*Macaca radiata*), and langur (*Presbytis entellus*) species are maintained at Central Drug Research Institute Lucknow, for biomedical research. The faecal samples were in general collected from those animals showing symptoms of diarrhoea before the commencement of any treatment.

ii) Rabbits : Faecal swabs from rabbits showing symptoms of gastroenteritis were examined for Isolation of *E. coli* strains.

iii) Poultry : Blood from septicaemic cases of laboratory fowl (Poultry Vaccine Institute, Badshah bagh, Lucknow) were examined for isolation of *E. coli* strains.

Indicator bacterial strains :Various standard indicator strains used in the present study are presented in Table-2.

ISOLATION AND IDENTIFICATION OF *E. COLI* ISOLATES :

Soon after collection, samples were streaked on MacConkey's agar plates and incubated aerobically at 37°C overnight. Four to five smooth, lactose fermenting colonies with entire regular edges, suspected to be *E. coli* were picked up, suspended in nutrient broth and culture was purified by repeated streaking on Mac-Conkey agar and Nutrient agar plates. A single colony was transferred to nutrient agar slant and was given an isolate number. The morphological, cultural, and biochemical characteristics were studied as described by Edwards and Ewing (1972), Breed *et al.* (1973) and Cruickshank *et al.* (1975).

A total of 11 biochemical reactions were studied to characterize Isolates of *Escherichia coli*. These are methyl red (MR) reaction, Voges Proskauer (VP) reaction, indole production, nitrate reduction, gelatin liquifaction, deamination of phenylalanine to phenyl pyruvic acid, utilization of citrate as sole source of carbon, production of H_2S and ammonia and hydrolysis of urea.

Eleven sugars were used to study fermentation behaviour of *E. coli* isolates. These sugars are lactose, sucrose, arabinose, xylose, rhamnose, raffinose, adonitol, inositol, dulcitol, sorbitol and salicin.

SEROLOGICAL TYPING:

The serological typing of isolates was done at National *Salmonella* and *Escherichia coli* Typing Centre, Central Research Institute, Kasauli,

Table2 : Reference / indicator bacterial strains under study

Sl. No.	Strains	Relevant genetic markers	Purpose	Source
1.	E. coli B (ECB)	Prototroph, non-plasmid bearing, non lysogenic, non colicinogenic, standard sensitive strain to colicins and antibiotics.	antibiotic potency control in MIC determination & for detection of colicin production	Central Drug Research Institue, Lucknow (India)
2.	E. coli NCTC-10418	Standard sensitive strain	Antibiotic potency control in antibiogram determination	Central Drug Research Institute, Lucknow (India)
3.	E.coli K-12 X^+	F^-, Lac^+, X^+ (mobilizing factor)	for mobilizing non-conjugable plasmids	Institute of Medical Sciences,B.H.U. Varanasi,(India).
4.	E. coli K-12 J62-I	F^-, lac^-, Pro^-, His^- ,Try^-, Nal^R	Recipient in conjugation experiments and also for detection of colicin production	Central Public Health Laboratory Colindale Avenue, London (U.K.)
5.	E. coli K-12/711	Toxin negative strain Nal^R, Lac^-, ST^-, LT^-	Negative control in toxin production assays	Dr. H.W. Smith, Poultry Research Station, Houghton(U.K.)
6.	E. coli P-16	O9:K9, ST^+	Positive control for ST toxin assay	do
7.	E. coli T-96	Serotype not known LT^+	Positive control for LT toxin assay	Dr. S.C. Pal, National Institute of Cholera and EntericDiseases Calcutta,(India).
8.	E. coli T-86	Setotype not know, ST^+ /LT^+	ST /LT control for toxin assay	do
9.	E. coli Row	Prototroph, Lac^+, sensitive to all colicins and antibiotics, non-lysogenic and non-colinogenic strain	For detection of colicin production	Central Drug Research Institute,Lucknow (India).

Himachal Pradesh (India). Somatic antigen ('O' groups) of these strains could be determined as antisera against somatic antigens only were available at this Centre.

BACTERIAL MEDIA USED :

Following media were used in the present study to maintain the cultures according to their requirement and nature of experiments conducted.

Nutrient Agar/Broth : All the strains were maintained on nutrient agar slants having peptone, 20 gm; sodium chloride, 5 gm; di-sodium hydrogen phosphate, 2.5 gm; dextrose, 2 gm; distilled water one litre at pH 7.2 with 2% agar. In broth agar was not added.

Mac Conkey's Agar Medium: It contains lactose, 10 gm; peptone, 20 gm; sodium taurohoclate, 5 gm; agar, 20 gm; distilled water, 1 litre; pH 7.2 and to this 3.5 ml of neutral red (2% solution of ethanol) was added as an indicator. MacConkey agar medium without bile salt was used in bacterial conjugation experiments for plasmid transfer studies.

Simmon's Citrate Agar : It was used for confirmatory testing of Salmonella typhimurium. It contains: NaCl, 5 gm; $MgSO_4$, 0.2gm; NH_4-H_2PO_4, 1.0 gm; KH_2PO_4, 1 gm; $Na_3C_6H_5O_7$ ($2H_2O$), 5 gm; agar, 20 gm; bromothymol blule, 40 ml. (0.2% solution) and volume was make up one litre with distilled water and adjusted pH, 6.8.

Minimal Synthetic Agar Medium : Confirmatory tests of *E. coli* K-12 trans-conjugants (for assessment of proline, histidine and tryptophane deficiency) were made on this medium having NH_4Cl, 1 gm; KH_2PO_4, 1.5 gm; $NaHPO_4$, 3.5 gm; $MgSO_4$ ($7H_2O$), 0.1 gm; lactic acid, 9 gm; agar 20gm and make up the valume 1 litre with distilled water, final pH was adjusted

at 7.2.

Colonisation Factor Antigen (CFA) Agar: CFA - agar was used as described by Evans *et al* (1979) for detecting adhering factors by slide haemagglutination of red blood cells of human type 'A'$^{+}$, bovine, sheep, rabbit, fowl and guinea pig. CFA - agar contains : Casamino acid, 1%; Yeast extract, 0.15%; $MgSO_4$, 0.005% and $MnCl_2$, 0.0005% and agar 2% at pH 7.4. It was autoclaved at 15 lb pressure for 10 minutes.

Medium used for detection of haemolytic activity of *E. coli* : (Blood Agar Medium) : Nutrient agar with 6% of defibrinated sheep blood was used for detection of haemolytic activity of strains. Sheep blood was collected aseptically in conical flask assembly with glass beads for removing fibrin to check the coagulation of blood.

Tryptic Soy Broth (TSB) : TSB has been used for enterotoxin production containing; trypton, 17 gm; soyton, 2.3gm; dextrose 2.5 gm; sodium chloride, 5 gm; di-potassium hydrogen phosphate, 2.5 gm; Yeast extract, 6 gm and distilled water one litre at pH 7.2. Medium was sterilized at 10 lb pressure for 15 minutes.

Yeast Extract Medium (YEM): YEM has been used for production of colicins. Medium contains: yeast extract, 2.5 gm; casein - hydrolysate, 2.5 gm; peptone, 15 gm; sodium chloride, 5 gm; dextrose, 2 gm; Na_2HPO_4, 1.5 gm; ground with distilled water upto one litre at pH 7.2.

ANTIBIOTIC SUSCEPTIBILITY TESTING OF *E. COLI* STRAINS:

(a) Disc diffusion method :

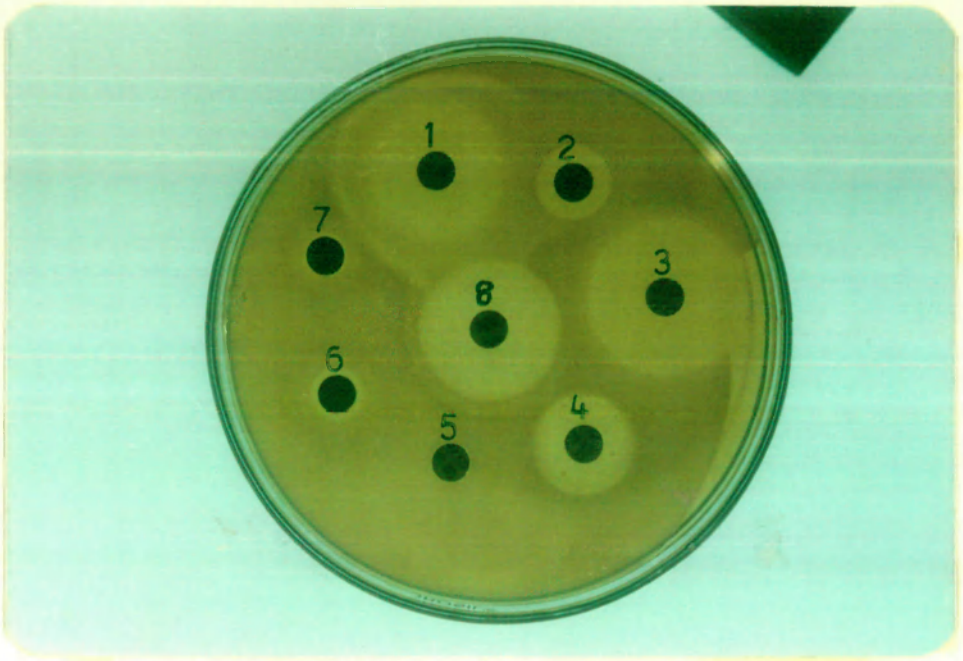
Antibiotic sensitivity behaviour of *E. coli* strains was preliminary determined by the disc diffusion method of Bauer *et al.*, (1966), using sensitive control strains *E. coli* B and *E. coli* NCTC 10418. The antibiotic discs were obtained from HI-Media Laboratories (India). The potency of discs was as follows :-

Ampicillin, Amoxycillin, Streptomycin (10 µg/disc each); Tetracycline, Doxycycline, Chloramphenicol, Kanamycin, Cotrimoxazole and Nalidixic acid (30 µg/disc each); Norfloxacin (10 µg/disc) and Nitrofurantion (300 µg/disc).

Nutrient agar plates were prepared by pouring sterile nutrient agar into petridishes. After the solidification of medium the plates tilted and dried before use at 37°C, to remove excess moisture from surface. Broth cultures were prepared by inoculating single colony of each strain from freshly sub-cultured petriplates into 5 ml nutrient broth and incubating the culture at 37°C for 3-4 hr to obtain moderate turbidity (10^5CFU/ml). A sterile cotton swab was dipped into diluted culture, excess fluid was removed from swab by rotating it at inner side of test tube wall and it was spread onto agar surface of petriplates. Plates were kept at room temperature for 10 minutes, mounted the antibiotic discs in and incubated the plate at 37°C for overnight. The plates were scored for resistance or sensitivity after 18 hr by comparing the chart based on the inhibitory zone diameter as given by the disc manufacturer. (Plate 1).

Plate 1: Showing antibiotic sensitivity pattern of a test E. coli isolate by Disc diffusion method. 1(Ampicillin), 2(Nitrofurantoin), 3(Chloramphenicol), 4(Cotrimoxazole), 5(Streptomycin), 6(Tetracy cline), 7(Doxycycline), 8(Nalidixic acid).

Plate 2: Master plate used for replica plating by sterile tooth pick.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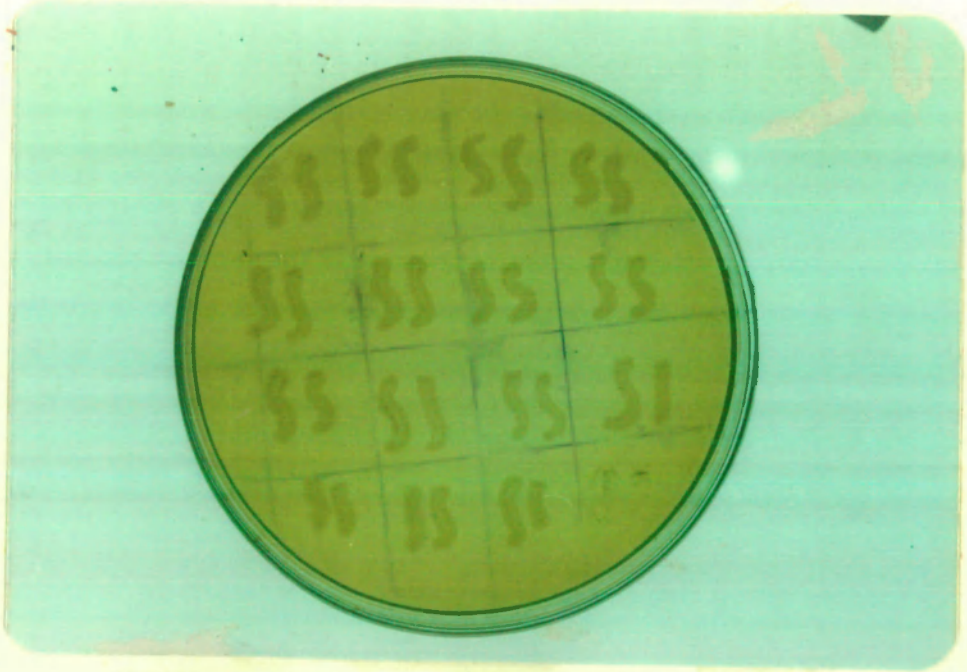


(b) Determination of resistance level by plate dilution method :

The minimum inhibitory concentrations (MICs) of individual drug was worked out by using following pure drugs powder obtained from their respective manufacturers: Ampicillin (Ap), Amoxycillin (Ax), Tetracycline (Tc), Doxycycline (Dx) and Streptomycin (Sm), (Hindustan Antibiotic Ltd. Pune India); Nalidixic acid (Nal) and Norfloxacin (Nr), (Ranbaxy laboratories, New Delhi, India); Chloramphenicol (Cm) (Park Davis); Kanamycin (Km) (Alembic Chemical Works) and Co-trimoxazolel (Co) was obtained from Burroghs-Welcome. All the drugs were soluble in water except Cm which was dissolved in 100% ethanol and then diluted in distilled water. Nal and Nr were soluble in alkaline pH (0.1 N NaOH) which was further diluted in distilled water.

Freshly prepared antibiotic solutions of different concentrations which were added to sterilized nutrient agar medium of pH 7.2, cooled to 45-50°C, mixed well and poured into petriplates (20 ml in each plate). The recommendations of WHO (1961) were followed in conducting the antibiotic sensitivity tests as adopted by Yadava and Gupta (1971).

Each plate was divided into twelve equal sectors. A loopful (5 mm diameter) of 18 hr. broth culture, diluted 10 fold in normal saline solution (NSS) was spot inoculated in duplicate in each sector on antibiotic medium plates, previously dried for an hr at 37°C. The control sets were also run parallely without any antibiotic. Inoculated plates were allowed to dry and incubated overnight at 37°C. The plates were examined for the presence or absence of growth on spotted area of antibiotic palates. The strains showing no visible growth on spotted area of the antibiotic plates but confluent growth on control plates without antibiotic were considered sensitive to that dilution. MIC of the antibiotics defined as the lowest

concentration per ml of medium that prevented visible growth of test strain. In each plate *E. coli* B was also spot inoculated in the centre as an antibiotic potency control.

Determination of MIC_{50} and MIC_{90} : MIC_{50} and MIC_{90} are defined as the MICs inhibiting 50% and 90% of the *E. coli* isolates respectively under test. Respective value has been calculated statistically.

GENETIC CHARACTERISATION OF DRUG RESISTANCE:

i) Detection of auto-transferable R-plasmids *in vitro* by conjugation :

Drug resistance transfer studies were carried out by broth mating technique as described by watanabe and Fukasawa (1961 b) and Datta and Nugent (1984), with little modification.

Donor strains : Strains resistant to one or more drugs isolated from human or animal diseases, served as resistant donors for antibiotic resistance transfer studies.

Recipient Strain : *E. coli* K-12 J62-1 (Nal^r, lac $^-$ Pro^-, His^-, Try^-) was used as recipient in transfer studies.

Mating procedure for conjugation studies : The donor and recipient strains were grown in 5 ml. nutrient broth and incubated for 3-4 hr. at 37°C. Donor and recipient growths were mixed in 1:2 ratio in 7 ml. fresh nutrient broth and incubated overnight at 37°C. Different dilutions of conjugate mixture were made in normal saline solution and 0.1 ml. mixture were then plated on to Mac Conkey's agar plates containing double antibiotics (100 µg Nalidixic acid and appropriate concentration of respec-

tive antibiotic to which the donor was resistant). At least 2 plates were used for each set of mixture. Plates were incubated at 37°C overnight and mixed broth were kept at room temperature. If no colony appears on selection plates, more plates were inoculated with mixtures that have been left at room temperature (28°C) day before, to look for a plasmid that is temperature sensitive for their conjugation function.

Confirmation of transconjugant clones : Non-lactose fermenting colonies appearing on selection plates (double antibiotic plate) were observed. They were identified and confirmed as recipient *E. coli* K-12 J62 on the basis of their cultural, morphological and nutritional characters. Colonies from each type of plates were replicated onto 3 sets of plate for confirming the recipient *E. coli* K-12 J62.

i) minimal agar (positive control)

ii) minimal agar + Pro, His, Try and nalidixic acid (negative control)

iii) minimal agar + Pro, His, Try + corresponding antibiotics.

Colonies appearing on 3rd set of plates were confirmed as K-12 J62 which acquired drug resistance plasmids.

ii) Detection of mobilizable R-Plasmids by Helper plasmid (*E. coli* X^+ factor) :

Non-conjugable R-determinants were attempted for mobilization by a standard mobilizing factor X^+ form *E. coli*-X^+ into a final recipient K-12 J62-I by a triparental cross technique as described by Anderson and Threlfall (1974). Over night broth culture of the donor, the mobilizing factor bearing strain (K12-X^+) and the final recipient K12J62 (0.5 ml each)

were mixed with 0.5 ml broth, incubated for 6 hr at 37°C, plated 0.1 ml of each and its dilutions (in NSS) on selection plates (Mac Conkey agar + Nal + corresponding antibiotic), and again incubated overnight at 37°C. Plates were scored for presence of colonies. The clones were confirmed by nutritional requirements (auxotrophy) of final recipient K-12J62 as described above. Such *E. coli* K-12J62 which acquired R-determinants of donor strain were considered positive for mobilization. Frequency of mobilization was calculated in terms of colonial count of transconjugants per final recipient *E. coli* K-12J62.

iii) Detection of plasmid mediated non-transferable drug resistance:

Plasmid elimination from the host strain by treating the cells with curing agents provide an additional information about the non-transferable plasmid mediated drug resistance. The method described by Watanabe and Fukasawa (1966 b) and Singh and Yadava (1988) was used. Three curing agents used are as follows :

i) Sodium dodecyl-sulphate (SDS)

ii) Acridine orange (AO)

iii) Norfloxacin (4- quinolone-compound)

Determination of MICs of curing agents: Minimum inhibitory concentrations (MICs) of curing agents were determined by plate dilution techniques as described previously for antibiotics then sub-inhibitory concentrations were taken to cure plasmid DNA selectively, as depicted in Table-3.

Curing Procedure: Nutrient broth, pH 7.6 was dispensed in tubes (5 ml each) and autoclaved. Various concentrations of sterilized curing

Table3: Curing Agents: Range of MICs and respective curing concentrations for the elimination of various plasmids.

Curing agents	Strain numbers	Range	
		MIC(µg/ml)	Selected curing conc. (µg/ml)
Norfloxacin (Nr)	75	0.05	0.02-0.05
	14,18,19,37,119,123	0.1	0.02-0.1
	2,3,4,11,115	0.2	0.04-0.2
	20,21,28	0.3	0.08-0.3
Acridine orange (AO)	115,119	100	50,100
	75	200	100,200
	3	400	100,200,400
	123	800	200,400,800,
	11	1600	400,800,1600
	19,20	2000	400,800,1600
Sodium dodecyl-sulphate (SDS)	119,123	9%	6%,8%,9%
	3,11,19,20,75,115	10%	6%,8%,10%

agents (except SDS which was added in nutrient broth prior to autoclaving) were added into tubes for eliminating the plasmid DNA from bacterial cell, Tubes were inoculated with 0.1 ml of overnight culture (diluted to 10^4 CFU/ml) and incubated at 37°C for 18 hr. Culture was appropriately diluted in NSS, 0.1 ml of it spread on nutrient agar, incubated overnight at 37°C. Resulting colonies were tested for antibiotic resistance by replica plating on nutrient agar plate incorporated with different antibiotics.

Control : Nutrient broth of pH 7.6 without curing agent was inoculated with the same organism to check the normal growth and for spontaneous loss of drug resistance markers, if any.

Procedure followed for replica plating : Replica plating method of Lederberg and Lederberg (1952), with slight modifications, was used for testing large number of colonies for presence or absence of R-determinants. A master plate was prepared by inoculating the isolated colonies with the help of sterilized tooth picks onto a fresh medium plate, previously numbered in sectors. Master plate was incubated overnight. Resulting colonies were replicated from master plate onto individual antibiotic plates in corresponding sectors with the help of a tooth pick for each colony. Plates were incubated over night and scored for presence or absence of R-determinants in each colony. (Plate -2).

DETECTION OF ENTEROTOXIN PRODUCTION AMONG *E. COLI* ISOLATES:

Culture conditions for production of enterotoxins :

Enterotoxin was prepared by inoculating a loop from active growth of each strain into 100 ml of TSB in 500 ml capacity Erlenmayer flasks followed by incubation at 37°C for 18-24 hr in rotary shaker at 200 rpm.

The culture was centrifuged at 10,000 Xg for 30 min at 4°C. Supernatant was collected and passed through a membrane filter of 0.45 mu pore size to obtain cell free filtrate (CFF).

This cell free supernatant was used as such for heat labile enterotoxin while for heat stable the same was heated at 65°-70°C for 30 min. This process destroys the LT enterotoxin leaving behind ST unaffected in the liquid. Crude filtrate thus prepared was used as such for LT or ST toxin throughout the study. Toxin was stored at -70°C in small aliquots until required.

Assays for the detection of heat labile (LT) and heat stable (ST) enterotoxin(s):

Guinea pig ileal loop assay (GILA) was performed essentially following the method of Saxena and Yadava (1982) and Choudhary *et al.* (1991) for detection of heat stable (ST) enterotoxins. Rabbit ileal loop assay (RILA) described by Moon and Whip (1971) as adopted by Yadava *et al.* (1988) was employed for the detection of heat labile (LT) enterotoxins. Briefly all the animals (4-5 month old guinea pigs and 6-8 month old rabbits) were fasted for 24 hr prior to assay. At the time of assay, animals were anesthetized, their abdomen opened aseptically and the small intestine (leaving 15 cm from the proximal and distal ends) was divided into 3-4 cm long loops by string ligature. Approximately 6-8 loops were prepared and one ml of each crude toxin preparation was inoculated into alternate loop. Intervening segments served as negative toxin controls. Non-toxigenic strain (*E. coli* K-12) was used as toxin negative control. For positive control, *E. coli* T-96 for LT and *E. coli* T-86 for (ST/LT) and P-16 for ST were taken. Negative and Positive toxin controls were included in each animals (Plates 3 and 4).

Table 4: Standard bacterial strains used for the assay of heat labile (LT) enterotoxin in Rabbit ileal loops

Reference strain designation	Type of enterotoxin	Heat treatment	Secretory response	Dilatation index after 18 hr. (SD*)
E. coli T-96	LT	a	+	1.93±0.32
		b	-	0.07±0.01
" T-86	ST/LT	a	+	1.81±0.14
		b	-	0.25±0.01
" P-16	ST	a	-	0.65±0.09
		b	-	0.41±0.07
" K12/711	Non-enterotoxi-genic	a	-	0.23±0.01
		b	-	0.28±0.03

* Mean value of 3 experiments
SD=Standard deviation
a= Un-heated; b = Heated
Minimum effective dose required to induce dilatation index 1.00 for LT toxin : 57.80 µg/ml (in terms of crude protein contents).

Table 5: Standard strains used for the assay of heat stable (ST) enterotoxins in Guinea pig ileal loops

Reference strains designation	Type of enterotoxin	Heat treatment	Secretory response	Dilatation index (±SD*) after 8 hr.
E. coli P-16	ST	a	+	0.61±0.04
		b	-	0.61±0.06
,, T-86	ST/LT	a	+	0.56±0.04
		b	+	0.57±0.03
,, T-96	LT	a	-	0.31±0.05
		b	-	0.09±0.05
,, K12/711	Non-enterotoxi-genic	a	-	0.09±0.05
		b	-	0.07±0.06

* Mean value of 3 experiments
SD=Standard deviation
a= Un-heated enterotoxin
b= Heated enterotoxin
Minimum effective dose of crude ST enterotoxin (in terms of protein contents) required to induce 0.5 D.I. for ST toxin: 31.25 µg/ml.

Plate 3: Dilatation of small intestine of guinea pig in response to *E.coli* heat stable enterotoxin after 8 hr. post inoculation.

O = Un-inoculated segment

+ = ST^+ *E coli.* P-16 strain

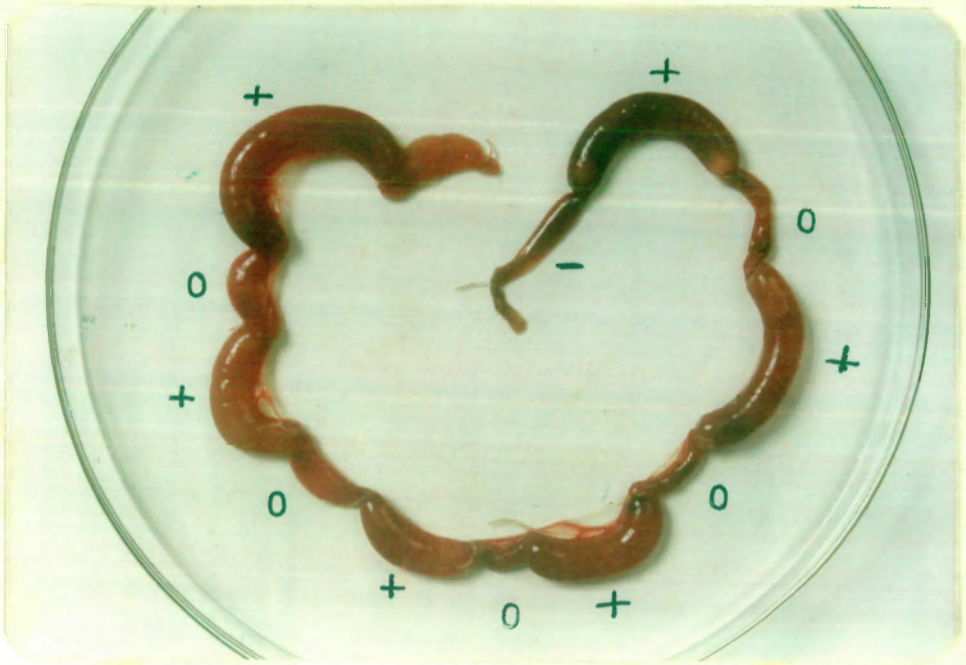
- = K12/711, a non toxigenic *E.coli* strain

Plate 4: Dilatation of small intestine of adult rabbit in response to *E. coli.* heat labile enterotoxin after 18 hr. post inoculation.

O = Un-inoculated segments

+ = LT^+ *E. coli* T-96 strain

- = K12/711, a non toxigenic *E.coli* strain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Secretory response in each loop was expressed in terms of dilatation index (DI). DI equal to 1.0 or more were taken as positive for LT toxin in RILA and 0.5 or more were considered positive for ST toxin in GILA. (Table 4 and 5).

$$DI = \frac{\text{Volume of fluid accumulated in one loop in ml.}}{\text{Length of the loop in cm.}}$$

Conjugal transfer of enterotoxin factors : Conjugation experiments was performed similarly used for conjugal transfer of R-plasmids (described earlier). The drug resistant *E. coli* transconjugant obtained from selection plates and confirmed on to minimal media were grown in Tryptic Soy Broth. Culture filtrate was prepared and tested for enterotoxins production in rabbit ileal loop model (for LT) and guinea pig ileal loop model (for ST) enterotoxins.

DETECTION OF COLICIN PRODUCTION :

Colicin production was studied according to the method described by Ansari and Yadava (1984). All the strains of *E. coli* were grown overnight in nutrient broth at 37°C. One ml of 18 hr old culture as added in 9 ml of yeast extract medium in a test tube and incubated for 30 minutes at 37°C. The content of the tube was poured in a petridish to form 2mm deep layer. The bacteria in petridish were exposed to ultra violet light for 2.5 minutes as standardized earlier at a distance of 25 cm from a portable Honovia Ultraviolet lamp (wavelength, 3000 A°), with a deep violet filter. The plate was placed on a sheet of card board and shaken during irradiation. After irradiation the content was poured in the tube and incubated for 38 hr at 37°C. Few drops of chloroform was added and shaken

vigorously for 15 seconds, left for 5 minutes at room temperature. The content was then centrifuged at 5000 rpm for 30 minutes. Chloroform treated supernatant as well as untreated 38 hr culture were spot inoculated with 5 mm diameter platinum loop over the lawn of three indicator strains (*E. coli* Row, *E. coli* B and *E. coli* K-12). The clear lysis or inhibition of indicator strain indicated presence of colicins (Plate 5). Sensitivity against *Salmonella typhimurium* strain was also done similarly.

To confirm the presence of colicin supernatant was diluted 2 fold serially and spot inoculated on the indicator strain *E. coli* Row by loop as described above. The intensity of inhibition of the indicator growth decreases and an end point was taken as the highest dilution at which a zone of inhibition was not clearly visible or hazy. This is differentiated from a lysogenic phage produced plaques because even at higher dilutions when there was only one virus particle the plaques or lysis will be clear on the indicator strains. On this basis colicin and phage (lysogenic) can easily be differentiated.

Conjugal transfer of Col-factors *in vitro* :

Colicinogenic strains were taken as donor and *Salmonella typhimurium* (Nal^r, $Citrate^+$) was taken as recipient. Conjugation experiments were performed similar as described for R-plasmid transfer studies. Two types of selection plates were used :

i) Nutrient agar + Nal (100 µg/ml)

ii) Nutrient agar$^+$ Nal (100 µg/ml) + corresponding antibiotics.

Transconjugants obtained on these selection plates were confirmed as *Sal. typhimurium* by citrate utilization. These transconjugants (100 No.)

were tested for colicin production by replica plate method, inoculating each clone to yeast extract medium in macrotitre plates (Plate-6). Plates were incubated over night at 37°C and then treated with chloroform, tested for colicin production onto their respective sensitive indicator strains).

Transconjugant obtained on selection plates were also screened for the transfer of one or more drug resistance markers. Stability of Col-factors: Transconjugants stored on master plate (without antibiotic pressure) were subcultured repeatedly and tested for stability of Col factors in their recipient *Salmonella typhimurium* strain (10 sub cultures).

SCREENING OF *E. COLI* STRAINS FOR HAEMOLYSIN PRODUCTION :

All the strains of *E. coli* under study were tested for their haemolysin production, employing erythrocytic agar plates using the method as described by Ahmad and Yadava (1980) and Blanco *et al.* (1990). Aseptically collected sheep blood was taken and 6% defibrinated blood were aseptically added in nutrient agar medium at 45°C, mixed by shaking without bubbles and plates were poured aseptically. On the other hands overnight growth of each strain was appropriately diluted in NSS, plated/streaked directly on blood agar plate, incubated for 24-48 hr or even more at 37°C to observe clear lysis (Plate - 7).

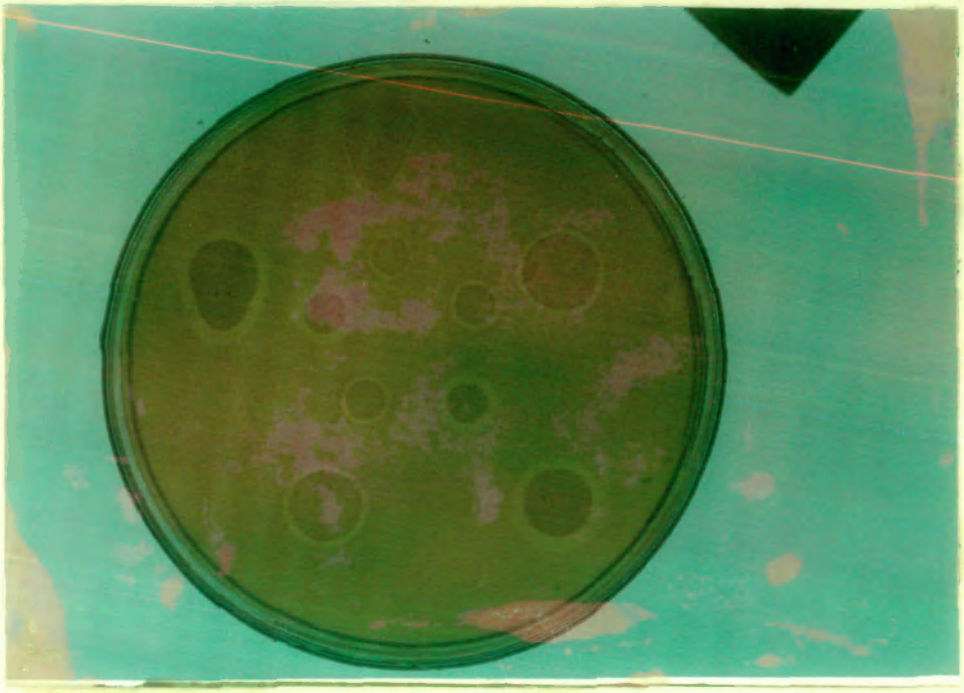
Detection of plasmid encoded haemolysins by conjugation *in vitro:*

The transfer studies of haemolytic factor were done according to Smith and Halls (1967) and LeMinor and LeCoueffic (1975). All the haemolytic strains were taken as donor, non-haemolytic strains resistant to nalidixic acid (to which donor strains were sensitive) were taken as

Plate 5: *Detection of colicin production by spot inoculation of test strains over the lawn of colicin sensitive E. coli ROW strain. (Zone of inhibition of indicator strain indicates production of colicin)*

Plate 6: Macrodilution plate used for testing colicin production of the large number of transconjugants obtained during transfer study of Col factors.

- = Uninoculated negative control well.



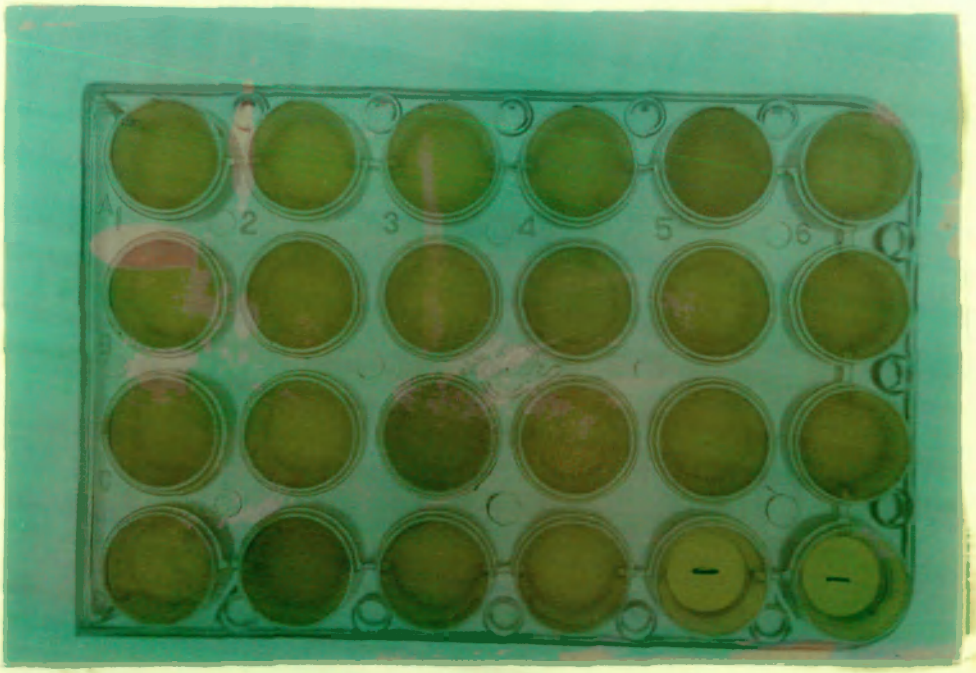

2
3
4
5
6
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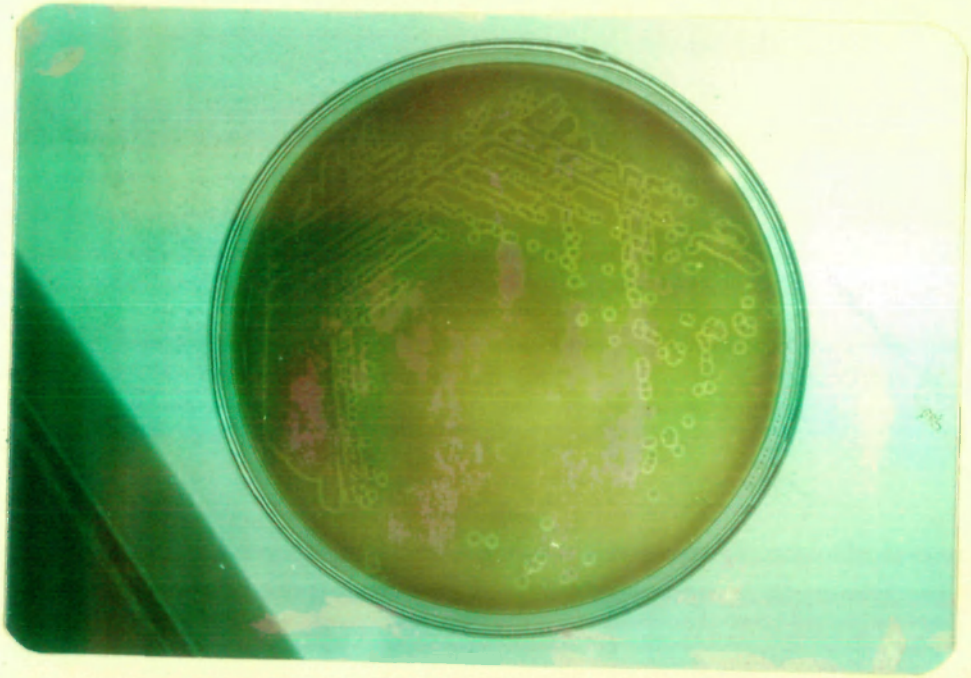
Plate 7: Showing zone of haemolysis on sheep blood agar plate of a haemolytic test strain (No. 23) after overnight incubation at 37°C.



recipient i.e. *Salmonella typhimurium*, *E. coli* K-12 J621 and *E. coli* PB-176.

The donor haemolytic strains and non-haemolytic recipient strains were conjugated in the almost same way as for drug resistance transfer studies. 0.1 ml. of each conjugant mixture and its dilutions were plated on blood agar plates containing 100 µg/ml of nalidixic acid, only recipient colonies could grow on such plates. Plates were incubated overnight at 37°C and examined for the presence/absence of haemolytic colonies. Confirmation of recipient haemolytic colonies was done by their morphological and nutritional characters.

Curing of haemolysin Factors : Procedure was same as for R-plasmid elimination studies, here blood agar plates were used instead of simple plates. Lack of haemolysin production was considered positive for elimination of HLY-factor.

Measurement of virulence and effect of HLY-plasmids on virulence of bacteria : The virulence of bacteria for mice was assessed by a modification of the test described by Vanden Bosch *et al.* (1979). Strains to be checked for virulence were grown overnight in nutrient broth and centrifuged at 5000 rpm for 30 minutes at room temperature, supernatant was removed, pellets washed twice in PBS. Pallets were resuspended in normal saline and a series of dilutions were made. Selected dilutions of know bacterial cells were injected intra peritoneally in different batches of mice. Each mice was inoculated with 0.1 ml. of each dilution, same amount spread over nutrient agar plate for counting the colonies. Morality was recorded upto ten days. A parallel batch of healthy control was also inoculated intraperitoneally with 0.1 ml. of normal saline.

Difference in virulence of two type of strains (plasmid bearing and non-plasmid bearing) was assessed by the mortality pattern of both strains in Swiss mice.

DETECTION OF ADHERING FACTORS INDIRECTLY BY SLIDE HAEMAGGLUTINATION TEST:

Haemagglutination tests were performed by slide agglutination for detection of the presence or absence of adhering factors. All the *E. coli* strains were grown overnight on CFA agar. Small amount of bacterial growth was taken and homogenized with small aliquot of normal saline. The bacterial suspension was then thoroughly mixed on glass slide with equal volume of diluted citrated blood samples. Fresh blood of human type A^+, fowl, sheep, bovine, rabbit and guinea pig was mixed with 3.8% sodium citrate solution in 9:1 ratio which was further diluted with phosphate buffer saline (PBS, pH 7.2) in 1:4 ratio. For mannose resistant haemagglutination 1% D-mannose was added in PBS solution. The mixture was mixed well and after 2 minutes haemagglutination was recorded.

Transfer of Adhering factor to recipient strains *E. coli* K-12 J 62-I:

E. coli K-12 J62-I was used as recipient strain for colonization factor transfer studies. It is a non-adhesive and non-agglutinating strain. Transfer studies were carried out in the same manner as in drug resistance transfer process. Hundred colonies of recipient from selection plates (100 µg/ml nalidixic acid) were checked separately for agglutination with erythrocytes of individuals of different species. Agglutinating colonies were supposed to receive the adhering factor.

RESULTS

BIOCHEMICAL AND SEROLOGICAL STUDIES OF *ESCHERICHIA COLI* STRAINS:

One hundred ninety four strains of *E.coli* were isolated from human (GIT,33; UTI, 30), monkeys (GIT, 97), poultry (Septicaemia, 24) and rabbits (GIT, 10) as depicted in Table 6. These 194 isolates were identified and characterised as *E.coli* on the basis of their cultural, morphological, biochemical and serological characteristics.

BIOCHEMICAL REACTION :

All 194 strains of *Escherichia coli* were studied for 11 biochemical reactions i.e., methyl red (MR) and Voges-Proskauer (VP) reaction, production of indole, ammonia, catalase, urease and hydrogen sulphide (H_2S) gas, reduction of nitrate to nitrite, liquefaction of gelatin, deamination of phenyl-alanine and utilization of citrate as sole source of carbon. Nearly all strains were found to be positive for production of acetyl-methyl-carbinol, nitrate reduction, ammonia and catalase production, negative for V.P. test, production of H_2S, urease, gelatinase and deamination of phenyl-alanine. However, four strains (No. 53,54,133,183) were negative for indole and one strain (No. 38) was found utilizing citrate as sole source of carbon (Table 7).

Eleven sugars (lactose, arabinose, xylose, sorbitol, rhamnose, dulcitol, sucrose, salicin, raffinose, adonitol and inositol) were used to study their fermentation behaviour. More than 95% strains fermented lactose, arabinose, xylose, sorbitol and rhamnose while adonitol and inositol were fermented by 5.6% and 1.0% strains respectively. Other 3 sugars (dulcitol, sucrose and salicin) were fermented by 43% to 66% of the strains (Table-8 a).

Table 6: Sources of 194 strains of Escherichia coli

Source	Diseased condition	Sample collected	Total strains	Strain designation
i) Human : a) Sporadic cases	Urinary-tract Infection (UTI)	Urine	30	1,3,4,5,6,7,9,10,12, 13,14,15,16,17,19, 20,21,22,23,25,26, 42,43,44,45,46,47, 48,49,50.
b)Sporadic cases	Gastroenteritis (GIT)	Stool	20	2,8,11,18,24,27, 28,29,30,31,32,33, 34,35,36,37,38, 39,40,41,
Laboratory attendants	GIT	Stool	13	134,135,136,137, 138,139,140,142, 143,144,145,146, 147.
ii) Animals : a) Monkey	GIT	Stool	84 13	51------134. 148-------160.
b) Poultry	Septiceamia	Blood	24	161-------184
c) Rabbit	GIT	Stool	10	185-----194
Total			194	

Table 7: Biochemical reactions of 194 strains of Escherichia coli				
Biochemical reactions	Positive strains		Negative strains	
	Number	Percentage	Number	percentage
Indole	190	97.92	4	12.08
Methyl red	194	100	0	0
Voges-Proskauer	0	0	194	100
Citrate	1	0.51	193	99.49
Ammonia	194	100	0	0
Catalase	194	100	0	0
Nitrate	194	100	0	0
Urease	0	0	194	100
Gelatinase(22°C))	0	0	194	100
Phenyl-alanine-deaminase	0	0	194	100
Hydrogen sulphide gas	0	0	194	100

Table 8a : Sugar fermentation reactions of 194 strains of Escherichia coli

Sugars	Positive strains				Negative strains	
	Acid	Acid & Gas	Number	%	Number	%
Lactose	1	189	190	97.92	4	2.08
Arabinose	0	193	193	99.49	1	0.51
Xylose	0	191	191	98.45	3	1.55
Sorbitol	0	187	187	96.39	7	3.6
Rhamnose	14	171	185	95.36	9	4.6
Dulcitol	0	129	129	66.49	65	33.50
Sucrose	29	93	122	62.88	72	37.11
Salicin	10	74	84	43.30	110	56.5
Raffinose	19	105	124	63.92	70	36.08
Adonitol	0	11	11	5.6	183	94.32
Inositol	0	2	2	1.03	192	98.97

Sugar fermentation reactions which were variable from strain to strain have been shown in Annexure-1. On the basis of heterogenous fermentation of 5 selected sugars (i.e. dulcitol, sucrose, salicin, raffinose and adonitol), 194 isolates of *E.coli* were grouped into 24 biotypes. The strains of biotype I utilised dulcitol, sucrose, salicin and raffinose with the production of acid or acid and gas. Biotype XXIV have only 5 strains which did not utilised any of these 5 sugars. The major biotype groups identified were biotype III and I having 50 and 33 strains respectively. Other main biotypes were No. VII (15 strains), XVI and XIX (14 strains each), VI (9 strains) as shown in Table 8 b. The strains from most of the major biotypes utilised mainly dulcitol, sucrose and raffinose in different combinations with other sugars.

SEROLOGICAL TYPING :

All 194 strains were confirmed as *E. coli* on the basis of their cultural, morphological, biochemical and sugar fermentation characters. Serological typing against only available somatic antigen (O-groups) was done at National *Escherichia coli* typing centre, Central Research Institute, Kasauli. (HP), India. Out of 194 strains, only 112 could be typed, while remaining 82 could not due to the limited availability of antisera. Typed 112 strains were distributed in 54 'O' serogroups. Maximum number of 6 strains were recorded in two 'O' serogroups, (O9 and 035 each) followed by serogroups, O2, O15 and O20 (five strains each); O25, O60, O68, O103 and O147 (four strains each); O1, O61, O73 and O84 (3 strains each); O7, O17, O22, O23, O38, O44, O70, O88, O93, O98, O143, O154 and O159 (two strains each) and rest 27 O-sero groups have only one strain in each group. (Annexure -2).

Incidence of various 'O' serogroups among *E.coli* strains of human

Table 8b : Classification of 194 E. coli isolates based on fermentation of 5 selected sugars

Biotype	Sugar fermentation pattern					Escherichia coli strains	
	DUL	SUC	SAL	RAF	ADO	Designation	Total no
I	AG	A/AG	A/AG	AG	-	11,13,14,15,22,25,33,34,35,37,52, 54,55,57,59,64,65,69,72,78,88,90, 100,104,105,117,120,151,155,156, 175,176,184	33
II	AG	AG	-	-	-	5,180	2
III	AG	A/AG	-	AG	-	1,2,4,7,8,10,12,16,18,27,28,29,30, 31,32,41,46,48,49,67,75,82,84,85,87 93,94,97,98,111,112,113,118,119, 121,124,128,137,139,143,147,148, 158,159,161,17 ,178,189,192,194	50
IV	AG	A	-	A	-	83, 181	2
V	AG	-	AG	AG	-	43,62,110,160,191	5
VI	-	A/AG	AG	AG	-	6,9,19,20,21,38,91,152,169	9
VII	-	AG	AG	-	AG	127	1
VIII	AG	A	AG	A	-	187	1
IX	AG	-	AG	-	-	17,51,53,56,68,71, 79,80,107,108 131,133,135,146,134	15
X	AG	-	-	AG	-	50,60,171,172,173	5
XI	-	AG	-	AG	-	42,141,153,154,165,166,	6
XII	-	A	-	AG	-	123,190,63	3
XIII	-	-	AG	-	AG	70,95,106,145,149	5
XIV	-	A	AG	-	-	103	1
XV	-	A	A	-	-	174,	1
XVI	-	A	A	AG	-	157	1
XVII	AG	-	-	-	-	3,77,87,99,101,115,116,122,132,13 142,162,163,170	14
XVIII	-	AG	-	-	-	24,26,36,44,92,168,179	7
XIX	-	-	A/AG	-	-	58,73,76,89,94,109,114, ,129, 130,136,138,140,150	13
XX	-	-	-	-	AG	66,81,96,125,126,	5
XXI	-	-	-	AG	-	23,39,45,186,188,162	6
XXII	-	A	-	-	-	164,183	2
XXIII	-	A	-	A	-	167,193	2
XXIV	-	-	-	-	-	40,61,86,182,185	5

- = No fermentation, A = Acid production, AG = Acid and gas production,
DUL = Dulcitol, SUC = Sucrose, SAL = Salicin, RAF = Raffinose, ADO = Adonitol

and non-human primates have been depicted in Table 9 a, which revealed that known human enteropathogenic 'O' serogroups. O2, O15, O20, O25, O44, O55 and O86 were encountered among monkey diarrhoeal isolates. In addition to these, serogroups O60, O73, O88 were also encountered both in human and monkey isolates (Table 9 b). Interestingly four (O1, O2, O44 and O159) known human pathogenic serotypes isolated from monkey have been reported here for the first time in India.

Occurrence of various pathogenic serogroups of *E.coli* was found to be associated mainly with the major biotypes and strains from such biotypes frequently utilised differential sugars (dulcitol, sucrose and raffinose) in different combinations. Thus, fermentation of these sugars were correlated with the pathogenicity of *E.coli* strains like pathogenic 'O' serogroups (Table 10).

ANTIBIOTIC RESISTANCE STATUS OF *E.COLI* STRAINS:

Preliminary screening of antibiotic sensitivity behaviour of *E. coli* isolates was conducted by disc diffusion method. All 194 strains of *E. coli* were also subjected to determine their minimum inhibitory concentrations (MICs) against eleven antibacterial drugs: viz., ampicillin, amoxycillin, tetracycline, doxycycline, streptomycin, chloramphenicol, kanamycin, co-trimoxazole, nitrofurantoin, nalidixic acid and norfloxacin. The disk diffusion method and MIC values were the criteria to check the antibiotic resistance pattern and resistance levels. Any strain was designated as resistant if that was given an inhibitory zone lesser than prescribed by respective disk manufacturers, or no inhibitory zone and such resistant strains showed its MIC value atleast >four folds of the MIC value of *E.coli*

Table 9 a : Incidence of somatic (O) antigen groups among Escherichia coli strains of human and non-human primates

Distribution of 'O' serogroups					
Monkey (GIT)			Human (GIT & UTI)		
O-serogroup	Total no. of O-group	Total number of strain	O-serogroup	Total no. of O-group	Total no of strain
O9,O35	2	12 (6 each)	O2	1	3
O15,O20,O103,O147	4	16 (4 each)			
O1,O25,O60,O61,O68		15 (3 each)			
O2,O7,O17,O22,O23 O38,O44,O70,O84 O93,O98,O154,O159	13	26 (2 each)	O73,O143	2	4 (2 each)
O32,O45,O51,O55 O57,O73,O80,O83 O88,O100,O101, O113,O115,O121,O145, O149,O156,O158	18	18 (1 each)	O4,O5,O12,O15, O20,O25,O36, O43,O50,O60, O68,O78,O88, O106,O128	15	15 (1 each)
Total	38	87		18	22

- Total number of typable strains = 112 (87, Monkey; 22, Human; 3, Poultry
GIT = Gastroenteritis, UTI = Urinary tract infection

Table 9 b : Common Occurrence of O-serogroups in E.coli isolates of man and animals origin

O- serogroup	Strains designation of		Total no. of strains
	Monkey (GIT) 87*	Human (GIT / UTI) 22*	
O2	117,127	46,139,147	5
O15	52,60,64,100	39	5
O20	59,101,110,126	134	5
O25	91,154,160	48	4
O60	58,88,156	44	4
O68	118,119,130	140	4
O73	69	137,144	3
O88	74	143	2
Total			32

* = Total number of typed strains

Table 10 : Occurrence of various O-serogroups among biotypes of E. coli strains.

Biotype	E. coli strain		O-serogroup
	Total	Serologically typed strain	
I	33	18	O8,O15,O20,O22,O23,O35,O44,O61, O84,O73,O93,O100,O103,O60,O115
III	50	26	O1,O2,O7,O9,O22,O25,O35,O36,O38,O50 O55,O68,O73,O88,O98,O147,O149,O156, O158
IX	15	12	O5,O9,O43,O57,O61,O88,O70,O147,O113
XVII	14	12	O7,O17,O20,O32,O73,O83,O103,O106,O145
V	5	3	O38,O20,O25
X	5	2	O4,O15
XI	6	3	O25,O44,O128
XVI	1	1	O101
XII	2	1	O93
XIII	5	4	O1,O43,O147,O154
VI	9	1	O25
XVIII	7	3	O35,O60,O127
XIX	14	14	O9,O12,O35,O45,O51,O60,O68,O143,O154 O159
XXI	5	1	O15
XX	5	5	O17,O20,O70,O103
XXII	2	1	O84
XXIII	2	1	O86
XIV	1	1	O84
XXIV	5	3	O23,O78,O80
IV	2	1	O61
IX	2	NIL	Non-typed
XV	1	NIL	
VIII	1	NIL	
VI	2	1	

B strain (an internationally accepted antibiotic sensitive strain which was isolated in pre-antibiotic era).

MICs of different antibacterial drugs against *E. coli* strains varied greatly. Very high level of drug resistance (> 1600 µg/ml) was recorded against Ap, Ax, Tc, Dx, Sm, Cm and co-trimoxazole (Annexure 3 and Table-11). It was observed that MIC_{90} for Ap, Ax, Tc, Dx, Sm and Cm was 50 µg/ml of each followed by Fd and Co (25.00 µg/ml each), Km (12.50 µg/ml), Nal (6.25 µg/ml) and norfloxacin (0.1 µg/ml) as depicted in Table-12.

Out of 194 strains of *E. coli* 111 (57.2%) were found resistant to one or more of the antibacterial drugs under test, while 42.78% strains were sensitive to all antibiotics. Resistance against single and double antibiotics was recorded nearly in same number of strains (18%) while majority, 70 (63.06%) of the strains were resistant to multiple (>3) drugs. Resistance to seven and even eight drugs simultaneously was also recorded among 4 and 3 strains respectively (Table-13). Over all incidence of drug resistance was found maximum for Tc (41.23%) followed by Sm (35.56%), Dx(31.95%), Ap(24.74%), Cm(20.61%), Ax (20.1%), Co(13.40%) and least for Fd (3.6%) and kanamycin (3.09%). None of the strains was found resistant to 4-quinolones (nalidixic acid and norfloxacin). Human strains showed more drug resistance (69%) as compared to animal isolates (54.0%) (Table 14).

AUTO-TRANSFERABLE R- PLASMIDS AMONG RESISTANT STRAINS OF *E.COLI* :

One hundred eleven resistant strains so obtained representing both human and animal sources were studied for the transferable or non-transferable nature of their drug resistance markers. Of these, only 35

Table 11: Minimum inhibitory concentrations (MICs) of 9 antibacterial drugs against 194 strains of Escherichia coli

Antibiotic (MICs-µg/ml)	Total number of strains								
	Ap	Ax	Tc	Dx	Sm	Cm	Km	Fd	Co
6400	5	6	0	0	1	0	0	0	0
3200	3	1	1	0	4	0	0	0	4
1600	15	10	1	1	5	3	0	0	4
800	5	9	3	1	5	7	1	0	3
400	4	3	12	6	8	14	0	1	1
200	3	4	20	13	21	10	0	0	6
100	3	0	24	17	12	6	1	4	6
50	2	2	13	16	9	0	4	2	2
25	7	4	6	8	7	0	4	46	8
12.5	18	9	21	24	92	24	50	102	106
6.25	42	29	13	11	22	42	33	36	54
3.12	75	49	41	16	5	43	36	2	0
1.56	12	64	34	57	3	30	63	1	0
0.78	0	4	5	24	0	12	2	0	0

Table 12: Susceptibility of 194 strains of Escherichia coli against various drugs

Antibacterial drugs	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Ampicillin	1.56-6400	6.25	50.00
Amoxycillin	0.78-6400	3.125	50.00
Tetracycline	0.78-3200	12.50	50.00
Doxycycline	0.78-1600	6.25	50.00
Streptomycin	1.56-6400	12.50	50.00
Chloramphenicol	0.40-1600	6.25	50.00
Kanamycin	0.78-800	3.125	12.50
Nitrofurantoin	3.125-400	12.50	25.00
Co-trimoxazole	6.25-3200	12.50	25.00
Nalidixic acid	0.4-25	3.125	6.25
Norfloxacin	0.02-4.0	0.025	0.10

MIC_{50}: Concentration lethal for 50% of the strains.
MIC_{90}: Concentration lethal for 90% of the strains.

Table 13: Drug resistance patterns of 194 strains of Escherichia coli

	Resistance profile	Strain designation	Total no.
Resistance to:	Sm	25,58,69,74,92,104,105	14
Single drug		112,113,134,138,139,144,147	
	Tc	81,120,124	3
	Ap	13,188	2
	Co	143	1
	Km	160	1
Double drugs	Sm,Tc	51	1
	Sm,Co	111,135	2
	Tc,Dx	3,8,48,63,94,101,158,168,175,16	10
	Tc,Co	148	1
	Ap,Tc	149,156	2
	Ap,Ax	34,47,62,72	4
Multiple :	Sm,Tc,Dx	50,53,66,68,71,80,86, 107,110,125,128,163	12
Three drugs	Sm,Tc,Co	22	1
	Sm,Tc,Fd	102	1
	Sm,Tc,Cm	116,121,123	3
	Tc,Dx,Co	24,70	2
	Tc,Dx,Km	159	1
	Ap,Ax,Sm	73,61,42	3
	Ap,Ax,Co	193	1
Four drugs	Ap,Ax,Sm,Tc	32	1
	Ap,Ax,Sm,Cm	4	1
	Ap,Ax,Sm,Co,	14	1
	Ap,Ax,Tc,Cm	11,46	2
	Ap,Ax,Co,Fd	18	1
	Sm.Tc,Dx,Cm	49,59	2
	Sm,Tc,Dx,Co	118	1
	Sm,Tc,Dx,Km	164	1
	Sm,Tc,Cm,Fd	103	1
	Tc,Dx,Cm,Co	26,28,36	3
	Ap,Sm,Dx,Cm	108	1
Five drugs	Ap,Ax,Sm,Tc,Cm	87	1
	Ap,Ax,Tc,Dx,Co	17,76	2
	Ap,Ax,Tc,Dx,Cm	2,37	2
	Ap,Sm,Tc,Cm,Fd	127	1
	Ap,Sm,Tc,Dx,Cm	41,82	2
	Ap,Sm,Tc,Dx,Co	23	1
	Sm,Tc,Dx,Cm,Co	48	1
	Sm,Tc,Dx,Cm,Km	40	1
Six drugs	Ap,Ax,Sm,Tc,Dx,Cm	7,12,45,85,95,109,119,146	8
	Ap,Ax,Tc,Dx,Cm,Co	19,21,30	3
	Ap,Ax,Tc,Tx,Cm,Km	39,44	2
	Ap,Ax,Sm,Tc,Dx,Co	194	1
Seven drugs	Ap,Ax,Sm,Tc,Dx,Cm,Km	60	1
	Ap,Ax,Sn,Tc,Dx,Cm,Fd	115	1
	Ap,Ax,Sm,Tc,Dx,Cm,Co	20,122	2
Eight drug	Ap,Ax,Sm,Tc,Dx,Cm,Co,Fd	38,132	2

Resistant strains = 111 (57.21%), Resistant to single drug = 21 (18.91%)
Resistant to two drugs = 20 (18.01%), Multiple resistant strains = 70 (63.56%)
Sensitive strains to all drugs tested = 83 (42.78%)

Table 14: Incidence of resistance against individual antibacterial drug among 194 strains of Escherichia coli isolated from various diseased conditions of man and animals.

Antibacterial drugs	Distribution of resistant strains in the following sources				
	Human (UTI & GIT) 63*	Monkey (GIT) 97*	Poultry Septicaemia 24*	Rabbit (GIT) 10*	Total number of resistant strains
Tetracycline	31 (49.20)	43 (44.32)	5 (20.83)	1 (10.00)	80 (41.23)
Streptomycin	23 (36.50)	43 (44.32)	2 (8.32)	1 (10.00)	69 (35.56)
Doxycycline	27 (42.85)	29 (29.89)	5 (20.83)	1 (10.00)	62 (31.95)
Ampicillin	26 (41.26)	19 (19.58)	Nil	3 (30.00)	48 (24.74)
Chloramphen-icol	23 (36.50)	17 (17.50)	Nil	Nil	40 (20.61)
Amoxycillin	23 (36.50)	14 (14.43)	Nil	2 (20.00)	39 (20.10)
Cotrimoxazole	16 (25.39)	8 (8.24)	Nil	2 (20.00)	26 (13.40)
Nitrofurantoin	2 (3.17)	5 (5.15)	Nil	Nil	7 (3.60)
Kanamycin	3 (4.76)	2 (2.06)	1 (4.16)	Nil	6 (3.09)
Nalidixic acid	Nil	Nil	Nil	Nil	Nil
Norfloxacin	Nil	Nil	Nil	Nil	Nil

* Total number of strains in respective source of Escherichia coli strains
In paranthesis % age is given

(31.53%) could transfer their resistance markers to recipient strain in different combinations, ranging from single to multiple or even en-bloc by *in vitro* conjugation at 37°C. The significant number of the transconjugants (n=16) were resistant to more than two drugs. Patterns of drug resistance transferred varied greatly in different strains irrespective of the drug resistance patterns of donor strains (Table-15, Figure-4). The overall incidence of conjugable r-determinants among 111 resistant strains of *E. coli* showed great variation. Maximum transfer was observed for Ax resistance marker (51.28%) followed by Ap(47.91%), Cm(27.51%), Co(26.92%), Tc(18.75%), Km(16.66%), Dx(14.51%), Fd(14.28%) and least for Sm(10.14%). (Table 16).

MOBILIZABLE (NON-CONJUGABLE) DRUG-RESISTANCE MARKERS :

Thirty five resistant strains representing various resistance patterns which had not shown the transfer of their drug resistance markers directly by conjugation were taken for mobilization studies with the help of conjugative plasmid (*E. coli* X^+ factor). Of these, only 6 (17.14%) strains could mobilize their antibiotic resistance markers partially or en-bloc. It was observed that resistance markers for Tc, Dx, and Ap/Ax could be frequently mobilized in varying frequencies (Table 17).

PLASMID ENCODED NON-TRANSFERABLE DRUG RESISTANCE :

To assess the curing efficacy and the curing dose of three curing agents (norfloxacin, acridine orange and sodium dodecyl sulphate) on R-factors, six strains possessing known transferable plasmids were subjected for curing experiments. Results showed that transferable R-factor was cured more efficiently with these curing agents whereas R-plasmid from one strain (No.18) could not be cured by any of these three curing agents. (Table-18 a). Further, to elucidate the nature of antibiotic resistance as

Table 15: Segregation of resistance markers during in vitro conjugation experiments from 111 donor strains to recipient E.coli K-12

Markers transferred to K-12		Original resistant patterns of donor strains	Strain designation	Total number
Patterns	Resistance profile			
Single drug	Km	Km	160	1
	Ap	Ap,Tc	149,156	2
	Sm	Sm,Tc,Dx	43	1
Double drugs	Ap,Ax	Ap,Ax	42	1
	Tc,Co	Tc,Co	148	1
	Tc,Dx	Tc,Dx	44	1
	Tc,Dx,	Tc,Dx,Km	159	1
	Tc,Dx	Sm,Tc,Dx	128	1
	Tc,Dx	Ap,Ax,Tc,Dx,Cm,Km	39	1
	Cm,Km	Sm,Tc,Dx,Cm,Km	40	1
	Ap,Ax	Ap,Ax,Sm,Tc,Dx,Cm	45,119	2
	Ap,Ax	Ap,Ax,Sm,Co	14	1
	Ap,Ax	Ap,Ax,Tc,Dx,Cm,Km	44	1
	Ap,Ax	Ap,Ax,Co,Fd,	18	1
	Ap,Ax	Ap,Ax,Tc,Cm	46	1
	Ap,Ax	Ap,Ax,Tc,Dx,Cm,Co	19,30	2
Three drugs	Sm,Tc,Cm	Sm,Tc,Cm	121,123,116	3
	Ap,Ax,Co	Ap,Ax,Co	193	1
	Ap,Ax,Cm	Ap,Ax,Sm,Tc,Cm	87	1
	Ap,Ax,Cm	Ap,Ax,Sm,Tc,Dx,Cm	95	1
	Ap,Ax,Co	Ap,Ax,Tc,Dx,Co	17	1
	Ap,Ax,Co	Ap,Ax,Sm,Tc,Dx,Co	194	1
	Ap,Sm,Cm	Ap,Sm,Tc,Dx,Cm	41	1
Four drugs	Ap,Ax,Sm,Tc	Ap,Ax,Sm,Tc	32	1
	Ap,Ax,Tc,Cm	Ap,Ax,Sm,Tc,Dx,Cm,Fd	115	1
	Tc,Dx,Cm,Co	Tc,Dx,Cm,Co	36	1
	Ap,Ax,Tc,Dx	Ap,Ax,Tc,Dx,Cm	12	1
≥Five drugs	Ap,Ax,Tc,Dx,Co	Ap,Ax,Tc,Dx,Co	76	1
	Ap,Ax,Tc,Dx,Cm, Fd	Ap,Ax,Sm,Tc,Dx,Cm,Co, Fd	132	1
	Ap,Ax,Sm,Tc,Dx, Cm,Co	Ap,Ax,Sm,Tc,Dx,Cm,Co	20	1
Total no. of strains showing tansferable R-plasmid				35 (31.53%)

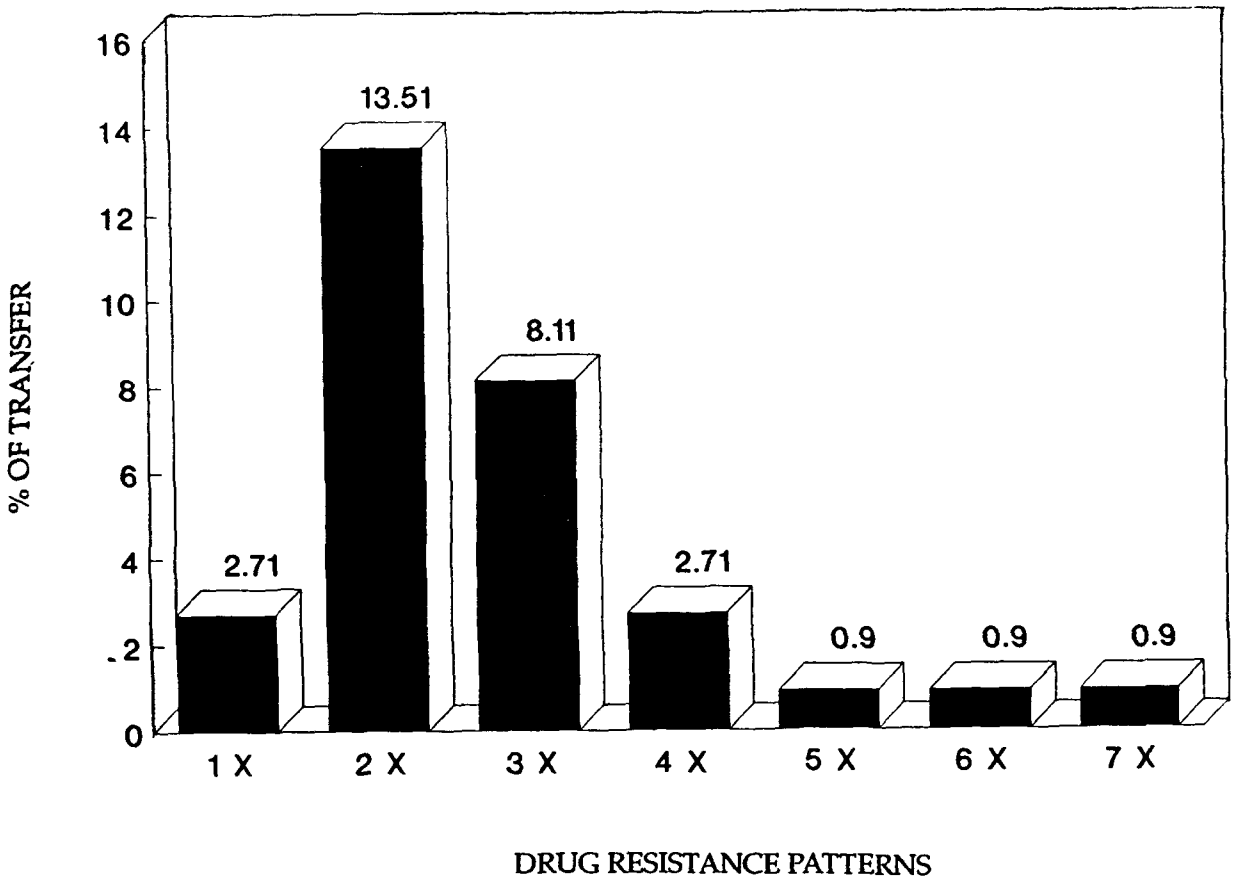


Figure 4 : Overall % transfer of single and multiple drug resistance in the strains of *E. coli*: Numbers 1X, 2X, 3X, 4X, 5X, 6X, 7X represent resistance to one, two, three, four, five, six and seven antibiotics respectively.

Table 16 : Prevalence of transferable r-determinants among resistant strains of Escherichia coli against individual drug

Drug resistance marker	Total no. of resistant strains	Strains showing transferable r-determinants	
		Total no.	Percentage
Amoxycillin	39	20	51.28
Ampicillin	48	23	47.91
Chloramphenicol	40	11	27.5
Co-trimoxazole	26	7	26.92
Tetracycline	80	15	18.75
Kanamycin	6	1	16.66
Doxycycline	62	9	14.51
Nitrofurantoin	7	1	14.28
Streptomycin	69	7	10.14

Table 17 : Mobilization frequency of R-plsmids from 6 non-conjugable Escherichia coli strains by a helper plasmid*

Parent non-conjugable strains		R-plsmid mobilization	
Designation	ResistanceProfile	r-determinants	Frequency
60	Ap,Ax,Tc,Dx,Sm,Cm,Km	Ap,Ax,Cm,Km	2.5×10^{-8}
85	Ap,Ax,Tc,Dx,Sm,Cm	Ap,Ax,Tc,Dx	2.9×10^{-7}, 2.1×10^{-7}
122	Ap,Ax,Tc,Dx,Sm,Cm,Co	Tc,Dx	1.4×10^{-6}, 1.5×10^{-6}
146	Ap,Ax,Tc,Dx,Sm,Cm	Ap,Ax	2.27×10^{-6}, 3.00×10^{-6}
73	Ap,Ax,Sm	Ap,Ax	2.00×10^{-7}, 2.5×10^{-7}
133	Sm,Co	Co	3.4×10^{-8}

* (Helper plasmid : E.coli X^{+} factor)

Table 18 a : Comparative evaluation of various curing agents for the elimination of transferable R-plasmids in 6 E. coli strains

Strains harbouring transferable plasmids		Cured resistance markers by various agents		
No.	Resistance Profile	Norfloxacin	Acridine orange	SDS
14	Ap,Ax,Sm,Co	Ap,Ax	Nil	Nil
18	Ap,Ax,Fd,Co	Nil	Nil	Nil
123	Sm,Tc,Cm	En-bloc	Eb-bloc	Tc,Cm
19	Ap,Ax,Tc,Dx,Cm,Co	Ap,Ax,Tc,Dx	Tc,Dx	Nil
115	Ap,Ax,Sm,Tc,Dx,Cm,Fd	Ap,Ax,Tc,Dx	Ap,Ax,Tc,Dx,C	Nil
20	Ap,Ax,Sm,Tc,Dx,Cm,Co	En-bloc	Ap,Ax	Ap,Ax

Table 18 b : Patterns of elimination of drug resistance markers by three curing agents among non-transferable drug resistant strains of Escherichia coli

Parent non-transferable strains		Cured resistance markers by various curing agents		
No.	Resistance Profile	Norfloxacin	Acridine orange	SDS
2	Ap,Ax,Tc,Dx,Cm	Ap,Ax,Cm	Nil	Nil
4	Ap,Ax,Sm,Cm	Ap,Ax,Cm	Nil	Nil
21	Ap,Ax,Tc,Dx,Cm,Co	Ap,Ax,Tc	Nil	Ap,Ax,Sm
11	Ap,Ax,Tc,Cm	Tc,Cm	Tc	Tc
3	Tc,Dx	Tc,Dx	Tc,Dx	Nil
28	Tc,Dx,Cm,Co	Cm	Nil	Nil
37	Ap,Ax,Tc,Dx,Cm	Nil	Nil	Tc,Dx,Cm

being chromosomal or plasmid mediated, we took 19 highly resistant strains showing non-transferable nature of resistance to treat with these 3 curing agents. It was observed that out of 19 resistant strains, 7 (36.05%) were cured one or more of their antibiotic resistance markers by the above used curing agents, thus plasmids could be confirmed in the above 7 strains. As compared to other curing agents, norfloxacin was found better curing agent for both transferable and non-transferable drug resistance R-plasmids (Table -18 b).

PLASMID ENCODED VIRULENCE FACTORS ASSOCIATED WITH *E.COLI* ISOLATES :

ENTEROTOXINS:

Of 194 strains 99 (51.03%) could produce either, ST, LT or both (ST/ LT) enterotoxins irrespective of their source of isolation. No significant differences in the incidence of ETEC strains among different sources was observed as the frequency of such strains ranged form 48.48 % to 54.61%. Interestingly, maximum number (74.12%) of toxigenic strains were producing heat stable enterotoxins followed by heat labile toxin producers (13.13%) and nearly same number of strains (12.12%) produced both ST as well as LT toxins. (Table 19, Figure 5).

Transferable nature of genetic determinants encoding biosynthesis of enterotoxins:

In the absence of any selection markers drug-sensitive enterotoxigenic strains could not be studied for their transferable nature of Ent-factors. Only drug resistant enterotoxigenic strains were studied using *E. coli* K-12 as recipient strain. Out of 58 R^+ ETEC strains 13 (22.41%) could transfer their enterotoxigenicity (Ent-factors) with drug resistance markers by conjugation. High transferability (41.44%) of Ent-factor was shown

Table 19 : Incidence of enterotoxigenic Escherichia coli (ETEC) strains among various pathological conditions of man and animals.

Source of strains	Disease condition	Total no. of strains	Types of Enterotoxins produced									Total number %
			Heat stable (ST)			Heat labile (LT)			ST/LT			
			Strain no.	Total	(%)	Strain no.	Total	%	Strain no.	Total	%	
Human	GIT	33	11,27,29,32,33 36,39,41,136, 141,143,146	12	(36.36)	18,40	2	(12.50)	34,38, 142	3	(18.75)	17 (51.51)
Human	UTI	30	3,6,10,23,43,49, 50	7	(23.33)	19,42	2	(16.66)	44,45,46	3	(25.0)	12 (40.0)
Monkey	GIT	97	52,53,54,56,58, 59,60,67,69,71, 73,74,79,80,84 85,86,87,88,91, 94,104,106,108, 110,113,116,118 123,127,128,130 132,149,154,156 157,160	38	(3826)	65,66, 72,75,76 98,120,158 159	9	(17.30)	62,78. 89,96 155,	5	(9.62)	52 (54.63)
Poultry	Speti-caemia	24	161,162,163,164 165,166,170,175 178,179,183,184	12	(50)	Nil	Nil	Nil	167	1	(7.14)	13 (54.61)
Rabbit	GIT	10	185,186,188 193,194	5	(50)	Nil	Nil	Nil	Nil	Nil	Nil	5 (50)
Total		194		74			13			12		99 (51.03)

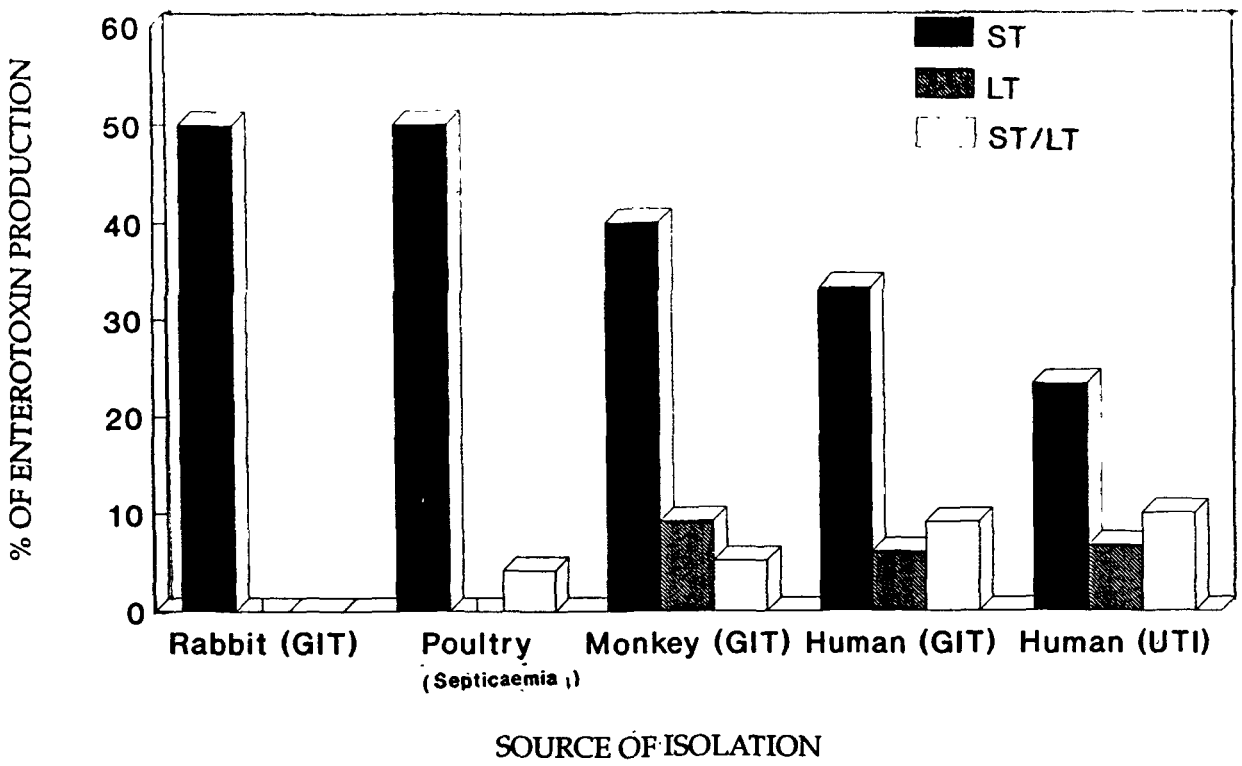


Figure 5: Comparative frequency of different enterotoxin production among the *E. coli* strains from human and various laboratory animal. (ST= Heat stable, LT= Heat labile enterotoxins)

Table 20: Co-transfer of antibiotic resistance and enterotoxigenicity factors from 58 R^+ ETEC strains to recipient strain E. coli K-12 by conjugation

Source/ Clinical condition	Antibiotic resistance factors transferred	Nature of enterotoxin transferred			Strain no.	Total
		LT	ST	LT/ST		
Human/GIT	Ap,Ax,Sm,Cm	-	+	-	41	1
	Tc,Dx	-	+	-	39	1
	Cm	+	-	-	40	1
Human/UTI	Ap,Ax,Sm,Tc	-	+	-	32	1
	Ap,Ax	-	-	+	44,46	2
	Ap,Ax	+	-	-	42	1
	Sm	-	+	-	43	1
Monkey/GIT	Sm,Tc,Cm	-	+	-	116,123	2
	Tc,Dx	+	-	-	159	1
	Tc	-	+	-	94	1
	Ap	-	+	-	156	1
Total		3	8	2		13

by human UTI isolates followed by isolates of human GIT (18.75%) and monkey GIT (18.75%) as depicted in Table-20.

INCIDENCE OF COLICIN PRODUCTION AMONG *E.COLI* STRAINS:

Out of 194 strains, 33 (17.01%) were found colicinogenic in nature as detected by *E. coli.* Row, while *E. coli B* and *E. coli* K-12 indicator strains could detect colicin production among 27(13.5%) and 22(11.8%) strains respectively (Table-21). The results showed that *E. coli* Row was the most suitable indicator strain for detecting the colicin production among *E. coli* strains. On the other hand *Salmonella typhimurium* was found to be resistant against all types of colicins, produced by 33 colicinogenic strains. Incidence of colicin production was higher among human strains as compared to strains from laboratory animals (Table-22-Fig. 6).

Infectious nature of colicin factors (Col-factors) :

As the indicator strains (*E. coli* B, *E. coli* K-12 and *E. coli* Row) were sensitive to various colicins, therefore, *Salmonella typhimurium* (which was resistant to all colicins) was taken as recipient strains for colicin transfer studies. Out of 33 colicinogenic strains of *E. coli* 17(51.51%) could transfer their Col-factors to *Salmonella typhimurium.* Higher percentage (66.66%) of transfer was found among human isolates followed by monkey isolate (44.44%). Frequency of transfer of Col-factors (as determined by replica plate method) showed great variation (16.6% to 100%). As regards the stability of Col factors, it was noticed that Col-factors of antibiotic sensitive donor strains were found more stable in the recipient strain than Col-factor of drug resistance donor strains. Nine transconjugants have stable Col-factors while 7 transconjugants lost their Col-factor after one to ten subcultures (less stable) as shown in Table-23.

Table 21: Screening of colicinogenicity among 194 strains of E. coli using 3 indicator strains

Colicin producing strains		Suceptibility of Indicator strains			
Designation	Total no.	E. coli Row	E.coli B	Ecoli K12	Sal. typhimurium
5,9,13,19,22, 32,33,37,39, 49,51,63,69, 80,85,99,100, 120,134,136, 148,150	22	+	+	+/±	-
2,23,27,126, 160	5	+	+	-	-
26,125,135, 151,165,192	6	+	-	-	-
Total	33	33 (17.01)	27 (13.91)	22 (11.34)	NIL

±/+ = Moderate/highly sensitive
\- = Resitant
In paranthesis % is given

Table 22: Incidence of colicin production among 194 E. coli strains of different clinical origin

Source	Clinical condition	Total no. of strains tested	Colicinogenic strains		
			Designation	Total	%age
Human	UTI	30	5,9.13,19,22, 23,26,49	8	26.26
Human	GIT	33	2,27,32,33,37, 39,136	7	21.21
Monkey	GIT	97	51,63,69,80, 85,99,100,120 125,126,134, 135,148,150, 151,160	16	16.49
Rabbit	GIT	10	192	1	10.00
Poultry	Septicaemia	24	165	1	4.16
Total		194		33	17.01

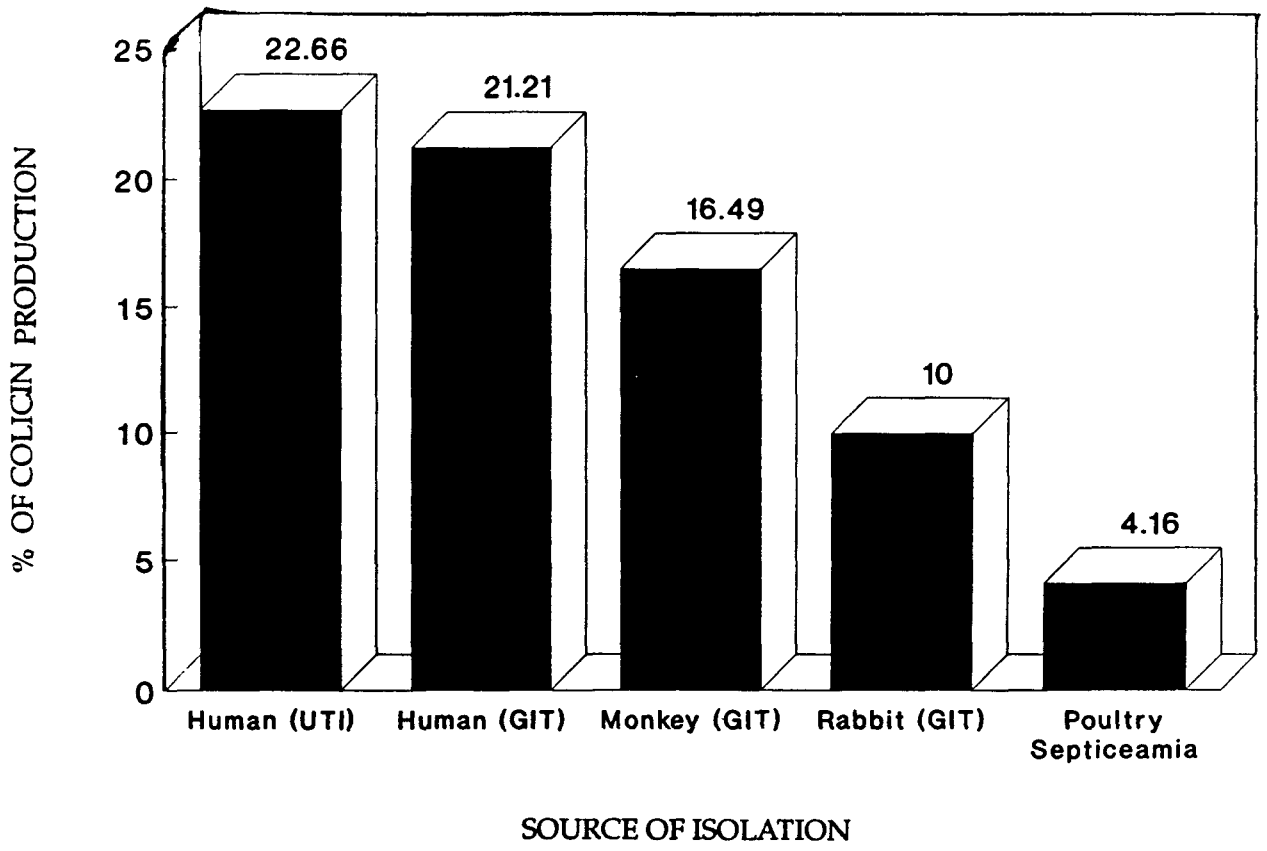


Figure 6 : Incidence of colicin production among the *E.coli* strains of clinical origin.

Table : 23 Inter-generic transfer of Col-plasmids and drug resistance markers form E. coli donors to Salmonella typhimurium recipient by conjugation

Colicin producing strains		Transfer of plasmids		Transferable Col-plasmids	
Designation	Resistance Profile	Col-plasmid	R-factor	*Frequency	**Stability
85	Ap,Ax,Sm,Tc,Cm	+	-	16.6	Not stable
2	Ap,Ax,Tc,Cm	+	-	16.6	Less stable
19	Ap,Ax,Tc,Cm	+	Ap,Ax	33.3	Less Stable
32	Ap,Ax,Tc,Sm	+	Ap,Ax,Sm,Tc	16.6	Not stable
23	Ap,Tc,Sm,Co	+	-	90	Stable
26	Ap,Tc,Sm,Co	+	-	33.3	Less stable
37	Ap,Ax,Tc,Cm	+	-	33.3	Stable
22	Tc,Sm	+	-	16.6	Not stable
120	Tc,Sm	+	Tc	100	Stable
80	Tc,Sm	+	-	33.3	Not stable
148	Tc,Co	+	Tc	100	Stable
63	Tc	+	-	90	Stable
134	Sm	+	-	16.6	Stable
5	Sensitive to drugs tested	+	NA	75	Less stable
27	"	+	NA	100	Stable
126	"	+	NA	90	Stable
137	"	+	NA	16.6	Stable

* Frequency of transfer has been calculated by replica plating mathod after screening the 100 clones.
\+ Tranfer of plasmid
\- No transfer
NA Not applicable
** Stability was checked for 10 subcultures at an interval of one month

HAEMOLYSINS :

All 194 strains of *E. coli* were screened for haemolysin production on 6% defibrinated sheep blood agar plate. Out of 194 strains only 27 (13.91%) could produce either α (21 strains) or ß (6 strains) haemolysins. Incidence of haemolysin production was found maximum among human UTI isolates (40.00%) followed by isolates of human GIT (12.12%), monkey GIT (10.30%) and poultry septicaemia (4.16%) as shown in Table-24 and Figure-7).

Extra-chromosomal nature of HLY-factors :-

The involvement of plasmids mediating haemolysin production was detected among 7 haemolytic strains. Of these, three strains No. 151, 153 and 160 could transfer their HLY-plasmids to *E. coli* K-12 or *E. coli* PB-176 by conjugation. Standard haemolytic *E.coli* strain P-233 transferred its haemolytic plasmid to the recipients *E. coli* K12 and *Salmonella typhimurium* (Table-25, Plate-8 and 9). On the other hand Hly-determinants had been cured from four strains No. 2, 4, 75 and 119 by one or more of the curing agents (norfloxacin, acridine orange and SDS) at there respective sub-minimal inhibitory concentration as described in Table-26.

Role of haemolysins in virulence and pathogenicity of *E. coli* strains:

All 27 haemolytic strains were injected intra-peritoneally at a dose of 0.17×10^8 CFU/mouse in different batches of mice, caused mortality of animals ranged from >20% to 100% (Table 27).

On the basis of their comparative Swiss mice mortality patterns these haemolytic strains of *E. coli* have been classified as virulent (70-100% mortality, 13 strains), partially virulent (20 -69% mortality, 12 strains) and avirulent (< 20% mortality, 2 strains). It was observed that majority of the haemolytic UTI strains were more virulent as compared to isolates causing

Table : 24 Haemolysin production among 194 strains of E. coli in various clinical conditions

Source (Clinical condition)	Total no. of Strains tested	Haemolysin producing strains		
		Designation	Total no.	Percentage
Human (UTI)	30	1,3,4,5,7,,9,10,21,23,26,49,50	12	40.00
Human (GIT)	33	2,24,28,30	4	12.12
Monkey (GIT)	97	60,69,75,119,151,152,153,154, 155,160	10	10.30
Poultry (Septicaemia)	24	163	1	4.16
Rabbit (GIT)	10	NIL	NIL	NIL
Total	194		27	13.91

Table 25: Inter and Intra-generic transfer of haemolysin factors (HLY) from haemolytic E. coli strains

Number of Haemolytic strains	Nature of haemolysin produced	Intra and Inter-generic transfer of HLY factor		
		*E. coli-K12	*E. coli PB-176	*Sal.typhimurium
1,2,3,4,5,9,23,24,26,28,30, 49,50,60,75,119,155,163	α	-	-	-
7,10,21,69,152,154	ß	-	-	-
151,153	α	+	-	-
160	α	+	+	-
P-233 (Standar strain)	α	+	-	+

\+ = Transfer of haemolytic factor;
\- = No transfer of haemolytic factor
* Recipient strains

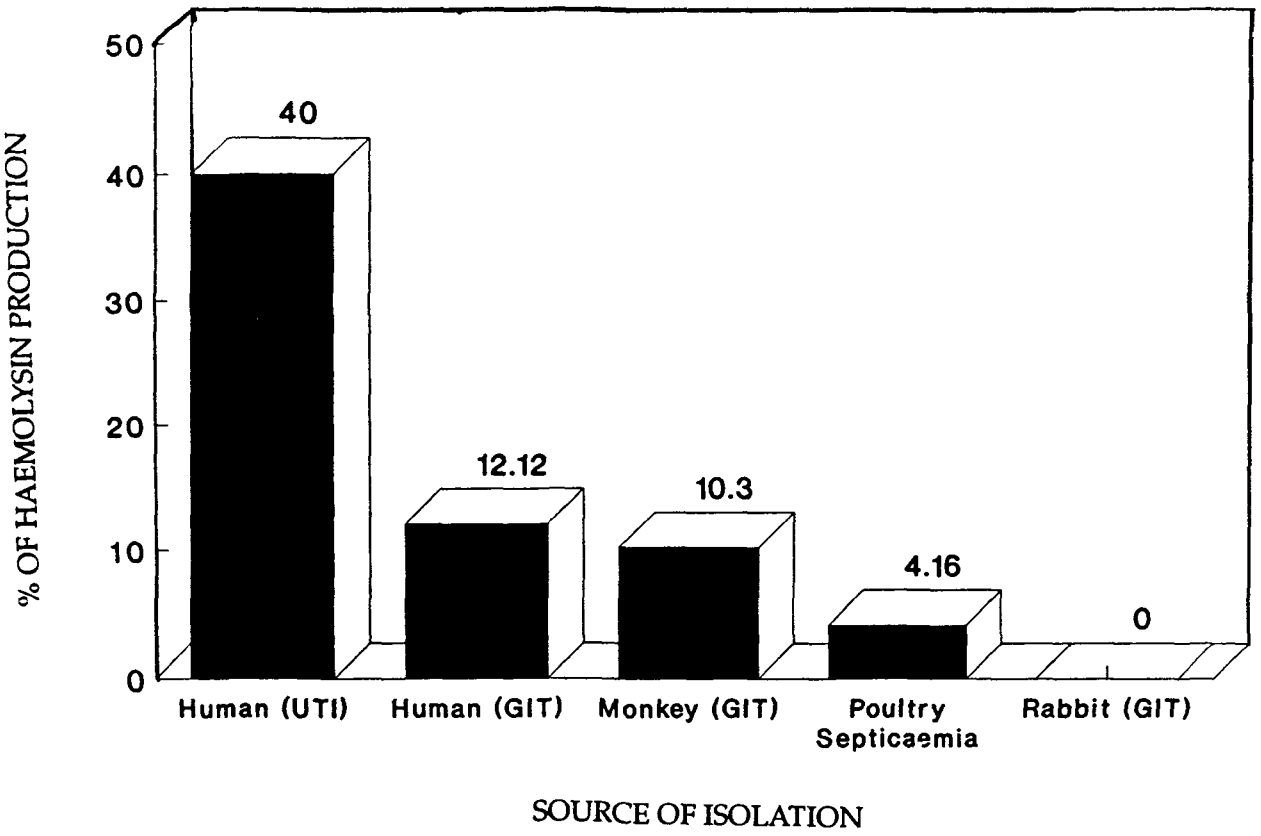
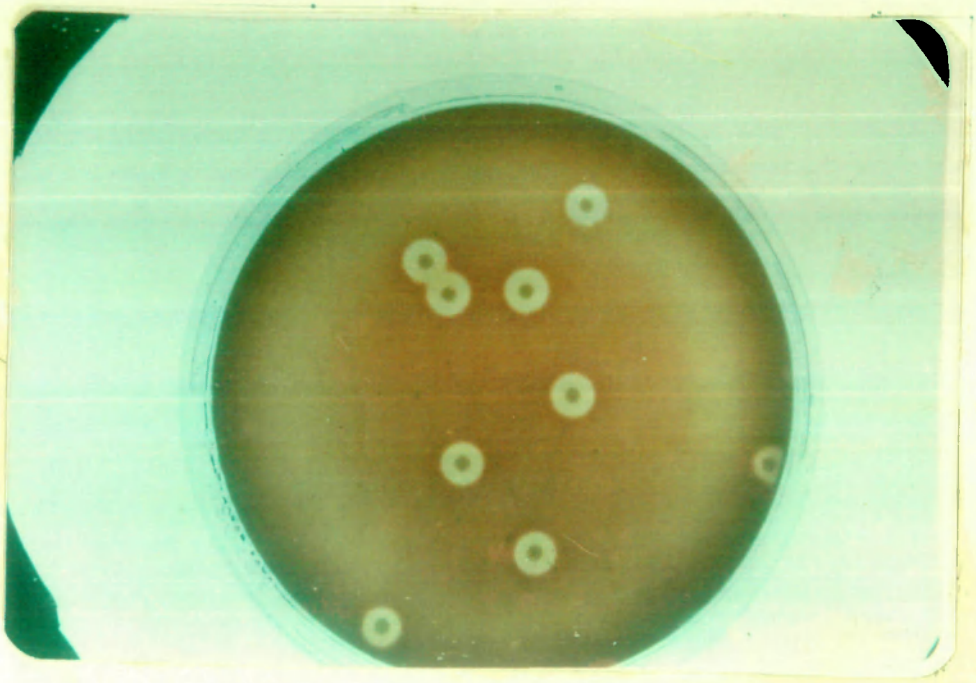


Figure 7 : Frequency of haemolysin production among the *E.coli* strains from man and animals.

Plate 8: Showing haemolysis by haemolytic *E. coli* strain (No. 151) on sheep blood agar plate.

Plate 9: Showing the haemolysin plasmid transfer from haemolytic strain *E. coli* 151 to recipient *E. coli* K-12 J62I.



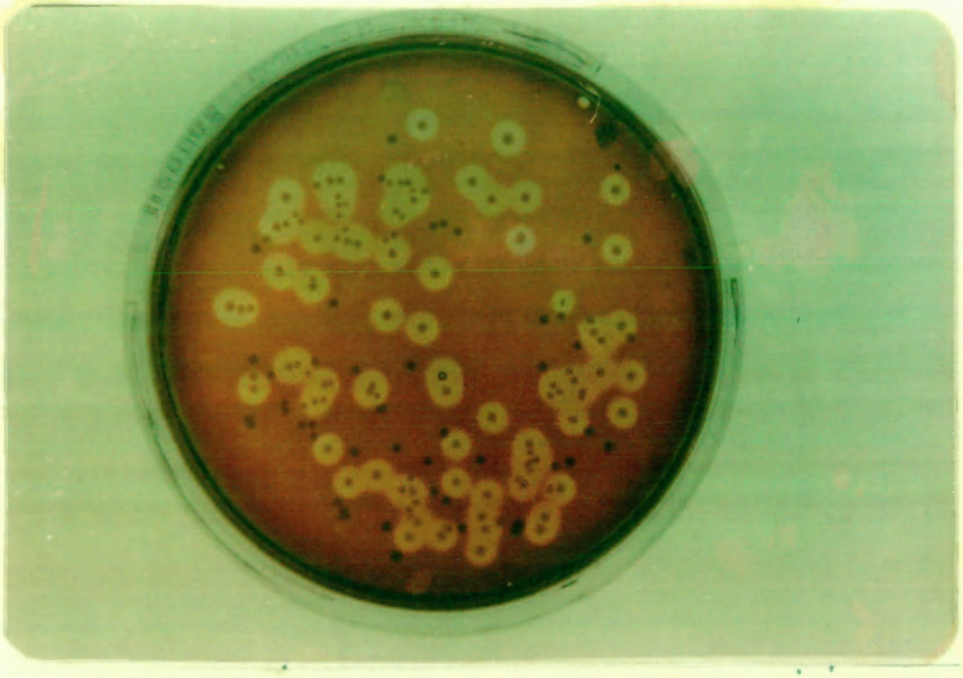


Table 26 : Elimination of haemolysin determinants from 24 Escherichia coli strains by norfloxacin and other curing agents

Haemolytic strain designation	Effect of curing agents		
	Norfloxacin	Acridine Organe	SDS
2	+	–	–
4	+	–	–
75	–	+	–
119	+	+	+
1,3,5,7,9,10,21, 23,24,26,28,30 49,50,60,69,152, 154,155,163	–	–	–

\+ = Haemolytic factor eliminated
\- = Haemolytic factor not eliminated

Table 27: An in vivo toxicity of haemolytic strains based on the mortality patterns in Swiss mice

Strain number	Mortality in days								% mortality
	0	1	2	3	4	5	6	7	
1,23,155	0/10	6/10	8/10	10/10	-	-	-	-	100.00
2	0/15	4/15	7/15	8/15	8/15	8/15	8/15	8/15	53.33
3	0/10	6/10	8/10	8/10	8/10	8/10	8/10	8/10	80.00
4	0/15	2/15	2/15	7/15	8/15	8/15	8/15	8/15	53.33
5	0/10	6/10	8/10	8/10	8/10	8/10	8/10	8/10	80.00
7,151	0/10	2/10	3/10	5/10	5/10	5/10	5/10	5/10	50.00
9,30	0/10	7/10	9/10	9/10	9/10	9/10	9/10	9/10	90.00
10,60	0/6	1/6	3/6	3/6	4/6	4/6	4/6	4/6	66.66
24,28	0/6	2/6	2/6	2/6	2/6	2/6	2/6	2/6	33.33
21,50,153	0/10	4/10	8/10	9/10	10/10	-	-	-	100.00
26,160	0/6	2/6	4/6	4/6	4/6	4/6	4/6	4/6	66.66
49	0/10	3/10	4/10	6/10	8/10	8/10	8/10	8/10	80.00
69	0/6	4/6	4/6	6/6	-	-	-	-	100.00
75	0/15	4/15	7/15	8/15	8/15	8/15	8/15	8/15	53.33
119	0/10	3/10	5/10	6/10	8/10	10/10	-	-	100.00
152	0.6	2/6	3/6	3/6	3/6	3/6	3/6	3/6	50.00
154	0/6	1/6	1/6	1/6	1/6	1/6	1/6	1/6	16.66
163	0/6	1/6	1/6	1/6	1/6	1/6	1/6	1/6	16.66

Inoculum size = 0.17×10^{8} CFU/mouse (Intra-peritoneal)

Table 28: An in vivo comparative evaluation of virulence of haemolytic strains based on mortality patterns in Swiss mice

Source/Clinical condition	Distribution of E.coli strains based on their respective mortality			Total
	Virulent	Partially virulent	Avirulent	
Human/UTI	1,3,5,9,21,23, 49,50	4,7,10,26	-	12
Human / GIT	30	2,24,28	-	04
Monkeys/ GIT	69,119,153, 158	60,75,151,152,160	154	10
Poultry/ Septicaemia	-	-	163	1
	13(48.14%)	12(44.44%)	02(7.40%)	27

70-100% mortality =Virulent
20-69% mortality = Partially virulent
<20% mortality = Avirulant

gastroenteritis in man and animals (Table 28)

To assess the virulence of haemolysin factors singly among *E.coli* strains, haemolytic strains and their homogenic strains after curing the haemolysin factor were further studied in Swiss mice. Each batch of 15 mice was given the dose of $0.17x10^8$ CFU/mouse intra-peritoneally. The mortality was observed upto seven days. The results showed that in all five homogenic Hly $^-$ derivative, the total percentage of mortality was reduced than that of their respective parent haemolytic strain. In *E. coli* 119 Hly$^+$ strain its homogenic non-haemolytic strain, mortality was reduced upto 40%. Other strains varied in the reduction of their mortality pattern (Table 29).

Similarly, four transconjugants of *E. coli* K12 so obtained (during conjugation experiments) and their respective donor haemolytic strains were studied. *E. coli* K-12 is non-haemolytic and non-pathogenic to mouse and does not cause any mortality in mice. A healthy control group of mice was inoculated with 0.2 ml of NSS/mouse. Results showed that patterns and percentage of mortality of 2 transconjugants (*E. coli* P - 233/K-12 Hly$^+$ and *E. coli* 151/K-12 Hly$^+$) conferred the same degree of virulence as that of their respective donor strains while, the mortality patterns of two other transconjugants (*E. coli* 160/K12 Hly$^+$ and *E. coli* 153/K12 Hly$^+$) was slightly less as compared to their donor strains. There was no mortality in control group (Table 30). Results showed that by transferring the HLY-plasmid to the recipient strain K-12 (non-haemolytic and non-pathogenic), the transconjugant becomes virulent which was well evident by mice mortality patterns. However, the virulence transferred by each donor haemolytic strain differ greatly in its degree of pathogenicity.

Table 29 : Comparision of the mortality of haemolytic and their corresponding homogenic non-haemolytic Escherichia coli strains in Swiss mice

Bacterial traits	* Mortality in days								% mortality
	0	1	2	3	4	5	6	7	
E. coli 2 HLY^+	0/15	4/15	7/15	8/15	8/15	8/15	8/15	8/15	53.33
E. coli 2 HLY^-	0/15	2/15	4/15	5/15	6/15	6/15	6/15	6/15	40.00
E. coli 4 HLY^+	0/15	2/15	5/15	7/15	8/15	8/15	8/15	8/15	53.33
E. coli 4 HLY^-	0/15	3/15	4/15	5/15	6/15	7/15	7/15	7/15	46.66
E. coli 75 HLY^+	0/15	1/15	2/15	5/15	8/15	8/15	8/15	8/15	53.33
E. coli 75 HLY^-	0/15	2/15	3/15	5/15	6/15	6/15	6/15	6/15	40.00
E. coli 119 HLY^+	0/15	4/15	8/15	8/15	12/15	14/15	15/15	15/15	100.00
E. coli119 HLY^-	0/15	2/15	4/15	6/15	9/15	9/15	9/15	9/15	60.00
E. coli P-233 HLY^+	0/15	5/15	5/15	6/15	6/15	6/15	6/15	6/15	40.00
E. coli P-233 HLY^-	0/15	2/15	3/15	4/15	5/15	5/15	5/15	5/15	33.33

* Mortality = No. of animals died/No. of animals inoculated
Inoculum Size : 0.17×10^8 CFU/mouse

Table 30 : Comparative mortality patterns of haemolytic donor and their E. coli K-12 transconjugants in Swiss mice

Traits of strain	** Mortality in days								% mortality
	0	1	2	3	4	5	6	7	
E. coli 160 HLY^+	0/6	2/6	4/6	4/6	4/6	4/6	4/6	4/6	66.66
K-12-160 HLY^+	0/6	2/6	2/6	2/6	3/6	3/6	3/6	3/6	50.00
E. coli153 HLY^+	0/6	4/6	6/6	6/6	6/6	6/6	6/6	6/6	100.00
K-12-153 HLY^+	0/6	0/6	1/6	2/6	4/6	4/6	4/6	4/6	66.66
E. coli 151 HLY^+	0/6	2/6	3/6	3/6	3/6	3/6	3/6	3/6	50.00
K-12-151 HLY^+	0/6	1/6	2/6	3/6	3/6	3/6	3/6	3/6	50.00
E. coli P-233 HLY^+	0/6	2/6	3/6	3/6	3/6	3/6	3/6	3/6	50.00
K-12 P 233 HLY^+	0/6	2/6	3/6	3/6	3/6	3/6	3/6	3/6	50.00
* E. coli K-12 HLY^-	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	Nil
* Healthy control	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	Nil

* Control
** Mortality = No. of animals died / No. of animals inoculated
Inoculum size : 0.17 X 10^8 CFU / mouse

HEAMAGGLUTININS (ADHERING FACTORS) ASSOCIATED WITH PATHOGENIC *E.COLI* STRAINS :

Detection of adhering factors indirectly by haemagglutination :

Slide haemagglutination was performed with the erythrocytes of human type A^+, rabbit, guinea pig, fowl, sheep and bovine against 194 strains of *E. coli.* Out of 194 strains 150 (77.32%) could express the presence of adhering factors as mannose sensitive haemagglutination (MSHA) 136 (69%) and mannose resistant haemagglutination 64 (33.5%) while, only 44 (22.68%) strains were found non-agglutinable with all test erythrocytes. These haemagglutinable *E.coli* strains showed various haemagglutinating patterns of MSHA or MRHA with one or more types of erythrocytes. It was also noticed that MRHA phenotypes encountered maximum among *E. coli* isolates of human origin as compared to animal isolates. Mannose sensitive haemagglutination was detected maximum against erythrocytes of guinea pig followed by fowl, rabbit and human type A^+, while mannose resistant was detected more against human type A^+ erythrocytes followed by fowl, sheep/bovine, guinea pig and rabbit. These observations have been presented in Table-31 and Table-32.

Further, on the basis of different haemagglutination behaviour, these 194 strains of *E. coli* were grouped into four major haemagglutinating phenotypes as $MSHA^+/MRHA^-$, $MSHA^+/MRHA^+$, $MSHA^-/MRHA^+$ and $MSHA^-/MRHA^-$ (Fig. 8). Results showed that an appreciable percentage (26.28) of *E.coli* strains showed both $MSHA^+/MRHA^+$ while majority (44.32%) of the strains showed $MSHA^+$ only. The incidence of $MRHA^+$ alone was recorded lowest (6.7%).

In an attempt to evaluate the involvement of transferable plasmids mediating adhering property, 21 haemagglutinating strains of UTI origin

Table 31: Haemagglutinating (adhering) ability of 194 E.coli strains with the erythrocytes of different species

Source of erythrocytes	Haemagglutination pattern		
	Mannose-sensitive (MSHA)	Mannose-resistant (MRHA)	Total no. of strains
Human Type A^+	35 (54.68)	29 (45.32)	64 (42.66)
Guinea pig	107 (96.39)	14 (12.61)	111 (74.00)
Fowl	76 (75.25)	25 (24.76)	101 (67.33)
Rabbit	75 (97.41)	2 (2.59)	77 (51.33)
Sheep	Nil	15 (100.00)	15 (10.00)
Bovine	Nil	15 (100.00)	15 (10.00)

* Total no. of bacterial strains showing haemagglutination with erythrocytes of one or more species = 150 (77.32%)
Non-haemagglutinating strains = 44 (22.68%)
In parenthesis %age is given.

Table 32: Distribution of various patterns of mannose sensitive haemagglutination (MSHA) and mannose resistant haemagglutination (MRHA) among E. coli strains of man and animals origin

Haemagglutination pattern		s Total no of haemagglutinating strains in their respective source of isolation				
	MSHA types	Human (UTI/GIT) 63*	Monkeys (GIT) 97*	Rabbits (GIT) 10*	Poultry (Septicaemia) 24*	Total No.
	R,G,F	8	8	0	8	24
	G,F	10	12	0	2	24
	R,G	2	12	2	1	17
	G	6	9	0	0	15
	F	8	6	1	0	15
	H,R,G,F	0	8	1	2	11
	H,R,G	1	4	0	4	9
	H,R	1	4	1	0	6
	H,G	1	3	1	1	6
	R	1	3	1	1	6
	H,R,F	0	2	0	0	2
	H,G,F	0	1	0	0	1
	Total (%)	38(60.31)	71(73.90)	7(70)	19(79.6)	136(69)
MRHA types	H	12	3	0	2	17
	F	5	2	2	7	16
	G	2	4	3	0	9
	H,S,B	3	2	0	0	5
	S,B	1	3	0	0	4
	H,F,S,B	2	1	0	0	3
	F,S,B	1	1	0	0	2
	G,F/H,F	2/2	0/0	0/0	0/0	2/2
	H,G/H,G,SB	1/1	0/0	0/0	0/0	1/1
	R	O	2	0	0	2
	Total (%)	32(50.79)	18(18.55)	5(50)	9(37.50)	64(33.5)

* Total number of E.coli isolates in their respective source of isolation
Sources of erythrocytes, S = Sheep; B = Bovine; H = Human type A; G = Guinea pig; F = Fowl and R = Rabbit

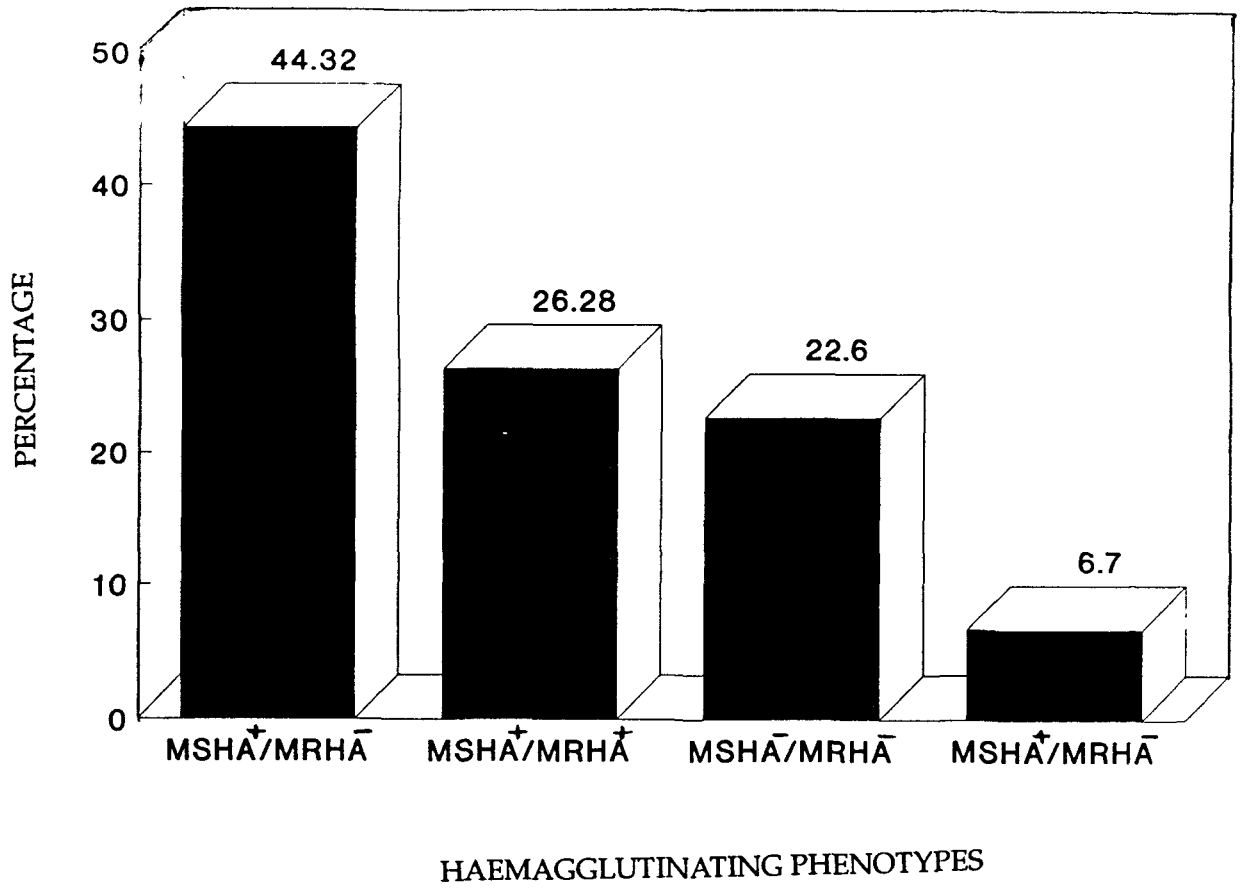


Figure 8 : Comparative frequnecy of different haemagglutinating phenotypes among the *E.coli* strains from man and animals.

Table 33 : Transfer frequency of adhering factors by conjugation among Escherichia coli strains of UTI origin.

Strain designation	Haemagglutination types		Transferred haemagglutinating patterns	Total No of strains
	MRHA	MSHA		
1	H,S,B	G,F	G,F	1
47	G	F	G	1
48	G	-	G	1
49	H,G	F	H,G	1
50		G,F	G,F	1

Total Tra^{+} strains = 5 (23.80%)
„ Tra^{-} strains = 16 (76.19%)
Sources of erythrocytes: H = Human type A, G = Guinea pig, S = Sheep, B = Bovine, F = Fowl

were studied by *in vitro* conjugation. Of these 5 (23.8%) strains could be transfer their adhering factor to recipient *E.coli* K-12 strain (Table 33).

STUDIES ON CORRELATION OF VARIOUS VIRULENCE FACTORS EFFECTING THE PATHOGENICITY OF *E. COLI* STRAINS

Association of various virulence factors such as enterotoxins, colicins, haemolysins and haemagglutinins (adhering factors) among 194 strains of *E. coli* were studied. The results of these studies are summarised as follows:

CORRELATION BETWEEN ADHERING FACTORS AND ENTEROTOXIGENICITY :

Out of 99 enterotoxigenic strains 79% possessed various adhering factors, (48.48% showed MSHA$^+$ reactions, 23.23%, MSHA$^+$/MRHA$^+$ and 8.07% showed MRHA reaction alone) as depicted in Table 34. Distribution of various haemagglutinating phenotypes among ST, LT and ST/LT toxins producing strains have been shown in Figure-9, which revealed that MSHA$^+$/MRHA$^-$ haemagglutinating phenotype occurred most frequently as compared to other phenotypes.

Haemagglutination patterns of human UTI and GIT isolates and their enterotoxigenic nature showed that 20 (71.42%) enterotoxigenic strains of human origin were positive for MSHA or MRHA reactions. Out of these 20 enterotoxigenic haemagglutinating strains 7 (35%) could show MRHA reaction with human type A$^+$ erythrocytes. Other strains showed different haemagglutination patterns as presented in Table-35

Table 34 : Association of enterotoxigenicity and haemagglutinating phenotypes among Escherichia coli isolates

Enterotoxin producing strains				Haemagglutinating phenotypes
ST	LT	ST/LT	Total no. of strain	
38	5	5	48	$MSHA^+/MRHA^-$
18	1	4	23	$MSHA^+/MRHA^+$
6	2	0	8	$MSHA^-/MRHA^+$
12	5	3	20	$MSHA^-/MRHA^-$

Total number of enterotoxigenic strains = 99
Total strains showing enterotoxin production and haemagglutination = 79
MSHA = Mannose-sensitive haemagglutination
MRHA = Mannose-resistant haemagglutination
+ = Positive reaction, - = Negative reaction

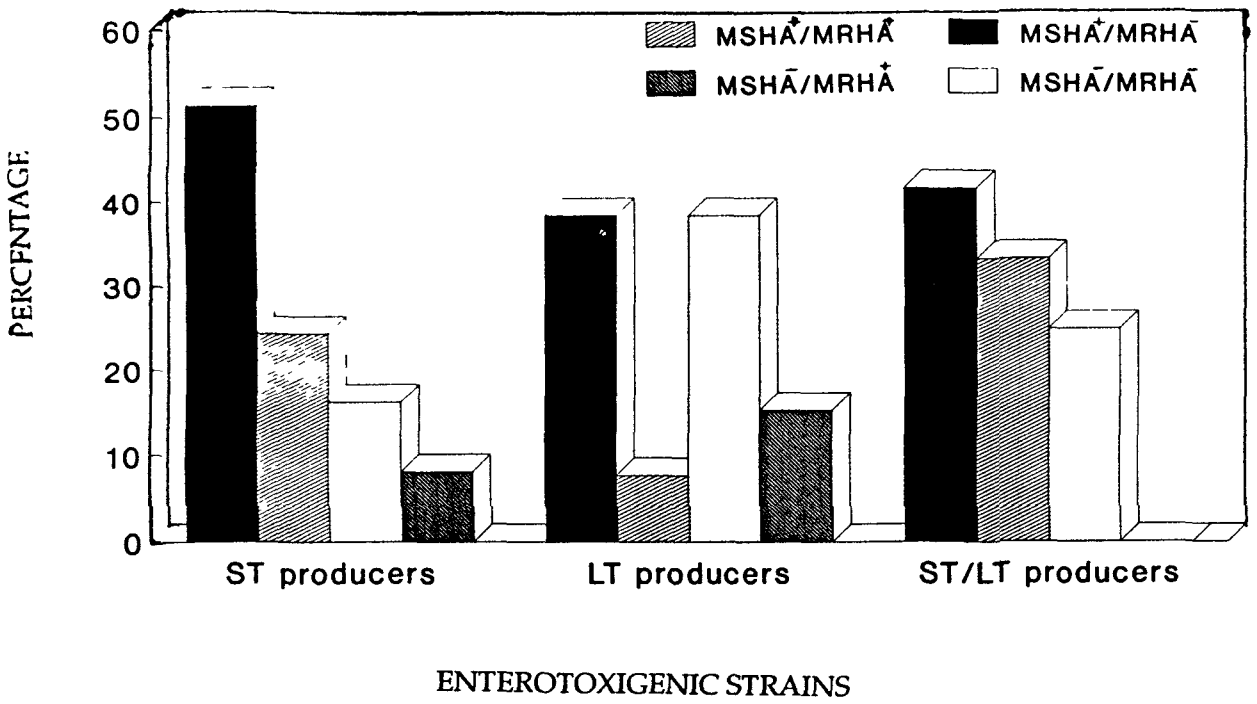


Figure 9 : Frequency of individual haemagglutinating phenotype among enterotoxigenic strains of *E. coli* (ST=Heat stable, LT= heat labile enterotoxins)

Table 35 : Haemagglutination patterns of enterotoxigenic E.coli (ETEC) strains of human origin in the presence or absence of D-mannose

Source and strain designation		Nature of toxin produced	Haemagglutination pattern with different erythrocytes	
UTI	GTI		MHSA	MRHA
50	32,36,143	ST	G,F	-
-	40	LT	R	-
42	-	LT	H	-
-	141	ST	G	-
44	-	LT/ST	F	-
3	-	ST	G	H,F,S,B,R
23	-	ST	G,F,R	H
-	27	ST	-	G,F
49	-	ST	G,F	H
-	33	ST	G,F,R	H
-	11	ST	-	F,S,B
-	29	ST	-	G,F
-	136	ST	-	S,B
-	146	ST	-	H
10	-	ST	G	-
-	34	LT/ST	-	H
-	142	LT/ST	G	H

Total ETEC strains showing haemagglutination with one or more erythrocytes = 20 (71.42%)

Sources of erythrocytes : H = Human type A^{+}, G = Guinea pig, R = Rabbit, S = Sheep, B = Bovine.

ASSOCIATION OF HAEMOLYSINS, ENTEROTOXINS AND ADHERING FACTORS (MRHA) AMONG *E. COLI* STRAINS:

Out of 27 haemolytic strains 12 (44.44%) were recorded enterotoxigenic in nature, maximum number of strains (10) were producing heat stable enterotoxin and of these 22 (81.48%) haemolytic strains could show mannose resistant haemagglutination (MRHA). Haemolytic strains of human UTI origin could show strong correlation with ST enterotoxin production and MRHA reaction while, haemolytic strains of human GIT origin could not produce enterotoxins but 50% of the strains showed MRHA reaction.

Interestingly, about 60% haemolytic strains of monkey origin were found to be enterotoxigenic and of these 90% demonstrated MRHA reactions (Table 36). Thus, a very strong correlation (compatibility) of haemolysins, ST enterotoxin, and adhering factors (MRHA) could be established among *E. coli.* isolates.

CORRELATION BETWEEN VARIOUS VIRULENCE FACTORS AND DRUG RESISTANCE AMONG *E. COLI* STRAINS :

Enterotoxins versus drug resistance factors:

A significant number (59%) of enterotoxigenic strains exhibited resistant to one or more of the drugs in various combinations. Prevalence of multiple drug resistance was observed among 65.51% of drug resistant ETEC strains. Among drug resistant strains, ST toxin producers were significantly higher as compared to LT and LT/ST producers (Table 37). These enterotoxigenic drug resistant strains showed good compatibility with drug resistance plasmids. Results of Table-38 revealed that among 48 R-plasmid harbouring strains, 28 (47.45%) could also harbour simultaneously the genetic determinants for enterotoxins. Out of these 28 enterotoxigenic drug resistance strains, 13 (46.42%) could transfer both

Table : 36 Correlation of haemolysins with enterotoxins and mannose resistance haemagglutination (MRHA), among 27 haemolytic strains of E. coli

Source/ Clinical condition	Haemolytic strain		Total no. of enterotoxin producing strain			Haemaggiutination pattern of HLY strain	
	no.	Total	ST	LT	ST/LT	MRHA	MSHA
Human (UTI)	1,3,4,5,7 9,10,21,24, 26,46,50	12	5	-	-	11	1
Human (GIT)	2,24,28,30	4	-	-	-	2	-
Monkey (GIT)	60,69,75, 115,119,152 153,154,155 160	10	4	1	1	9	1
Poultry (Septica-emia)	163	1	1	-	-	-	1
Total		27	10	1	1	22	3

Table 37: Correlation of enterotoxin production and drug resistance behaviour of Escherichia coli strains of man and animal origin

Sensitivity behaviour	Resistance profile	Enterotoxins producing strains designation			
		ST	LT	ST/LT	Total
Resistant to : single drug	Sm	58,69,73,74, 104,113	Nil	Nil	6
	Tc	Nil	120	Nil	1
	Ap	188	Nil	Nil	1
	Co	143	Nil	Nil	1
	Km	160	Nil	Nil	1
Double drugs	Tc,Dx	50,94,175,	158	Nil	4
	Ap,Ax	Nil	42,72	34,64	4
	Ap,Tc	149,156	Nil	Nil	2
Three drugs	Ap,Ax,Co	193	Nil	Nil	1
	Ap,Tc,Cm	116,123,128,163	Nil	Nil	4
	Sm,Tc,Dx	43,53,71,80,86,110	Nil	Nil	6
	Sm,Tc,Co	Nil	66	Nil	1
	Tc,Dx,Cm	3,59	Nil	Nil	2
	Tc,Dx,Km	Nil	159	Nil	1
Four drugs	Ap,Ax,Tc,Cm	11	19	46	3
	Ap,Ax,Sm,Tc	32	Nil	Nil	1
	Ap,Sm,Cm,Fd	127	Nil	Nil	1
	Ap,Sm,Tc,Cm	108	Nil	Nil	1
	Ap,Tc,Dx,Co	23	Nil	Nil	1
	Tc,Dx,Cm,Co	36	Nil	Nil	1
	Sm,Tc,Dx,Co	118	Nil	Nil	1
	Sm,Tc,Dx,Cm	49	Nil	Nil	1
	Sm,Tc,Dx,Km	164	Nil	Nil	1
	Ap,Ax,Co,Fd	Nil	18	Nil	1
>Five drugs	Ap,Ax,Sm,Tc,Cm	87	Nil	Nil	1
	Ap,Sm,Tc,Dx,Cm	41	Nil	Nil	1
	Ap,Ax,Tc,Dx,Co	Nil	76	Nil	1
	Sm,Tc,Dx,Cm,Km	Nil	40	Nil	1
	Ap,Ax,Sm,Tc,Dx,Co	194	Nil	Nil	1
	Ap,Ax,Sm,Tc,Dx,Cm	85,146	Nil	45	3
	Ap,Ax,Tc,Dx,Cm,Km	Nil	Nil	44	1
	Ap,Ax,Sm,Tc,Dx,Cm	16,132	Nil	38	3
	Ap,Ax,Sm,Tc,Dx,Cm,Kmm	60	Nil	Nil	1
Sensitive to all drug tested	Sensitive to all drugs	6,10,27,29,33,52,54,56,75,67 ,79,84,88,91,106,130,136,141, 154,157,161,162,165,166,170, 178,179,183,184,185,186	65,75,98	78,89, 96,142, 155, 167	39
Total no. of drug resistant and drug-sensitive enterotoxigenic strains		74	13	12	99

Total no. of ETEC strains resistant to single drug - 10, double drugs - 10, > three drugs - 39,

Table 38: Co-transfer of R-Plasmids and genetic determinants coding for biosynthesis of enterotoxins.

Nature of drug resistance of E. coli strians	Total number of resistant strains	Total number of R^+ETEC strains	Total no. of ETEC, harbouring Tra^+ Ent and R factors
Conjugable with E. coli K-12	35	24 (68.57)	13 (54.16)
Mobilizable by helper plasmid	6	3	Nil
*Non-conjugable and non-mobilizable plasmid bearing strains	7	1	Nil
Uncharacterized drug resistance determinants	63	31 (52.54)	Nil
Total	111	59	13

R^+ETEC strains = 28(47.45%)
Tra^+ R^+ETECstrains 13(46.42%)
Figures in parenthesis indicate percentage
* curable by one or other curing agents.

their genetic markers for drug resistance and enterotoxin simultaneously by conjugation to the recipient strain *E. coli* K-12.

Correlation between colicins production and drug - resistance among *E. coli* strains :

An appreciable number (57.57%) of colicinogenic strains were resistant to one or more of the drugs tested. Four strains (No. 19, 32, 120, 148) transferred their R-factor and Col-factor simultaneously. Out of 19 Col^+ drug resistant strains, 7 (No. 2, 19, 32, 37, 39, 85, 148) possessed R-plasmid which was proved by conjugation, mobilization and curing experiments. Interestingly, 68.42% of the Col^+ R^+ strains transferred their Col-factor by conjugation whereas, the drug sensitive colicinogenic strains could transfer their Col-factor to the extent of 26.26% only. Thus a high affinity was recorded between these two plasmids encoded characters to co-exist in *E. coli* strains (Table 39).

Haemolysins production and its affinity with drug resistance :

Out of 27 haemolytic strains, 17 (62.8%) were found resistant to one or more drugs in different combinations. Out of these, 17 haemolytic drug resistant strains, 8 strains possessed R-plasmids. However, co-transferability of haemolysin genetic determinants and drug resistants marker was observed only in one strain (No. 160). Genetic markers for haemolysin production and drug resistance could also be co-eliminated among 3 strains (No. 2, 4, 119) by one or other curing agents. These results indicated the affinity of Hly-determinants with R-plasmids to co-exist among *E. coli* strains (Table 40).

Correlation of adhering factors (haemagglutinin factors) and drug resistance:

A high (68.18%) incidence of adhering factors (MSHA/MRHA) was recorded among drug resistant strains, indicated a strong correlation between these two factors among *E. coli* population (Table 41).

Table 39 : Correlation between drug-resistance and colicinogenicity of *Escherichia coli* population

Colicinogenic strains				
Sensitivity pattern	Designation	Total	Transferring factors	
			Col	Col + R
Resistant to one or more drugs	2,13,19,22,23,26, 27,32,39,49,51,63, 69,80,85,120,125 134,148	19 (57.57)	13 (68.42)	4 (30.76)
Sensitive to all durgs tested	5,9,27,33,99,100, 126,135,136,150, 151,160,165,192	14 (42.42)	4 (26.26)	NA
Total		33	27	

Figures in parenthesis indicate percentage
NA- Not applicable

Table 40 : Association of haemolysin production with drug resistance plasmids

Haemolytic Escherichia coli strains			
Resistance profile	Designation	Presence of plasmids coding for	
		Haemolysin	Drug resistance
Sm	69	NC	NC
Km	160	HLY*	R*
Tc,Dx	3	NC	R
Sm,Tc,Dx	50,163	NC	NC
Tc,Dx,Co	24	HLY	R
Tc,Dx,Cm,Co	28	NC	NC
Tc,Dx,Cm,Co	26	NC	NC
Ap,Tc,Dx,Co	23	NC	NC
Sm,Tc,Dx,Cm	49	NC	NC
Ap,Ax,Sm,Cm	4	HLY	R
Ap,Ax,Tc,Dx,Cm	2	HLY	R
Ap,Ax,Sm,Tc,Dx,Cm	7	NC	NC
Ap,Ax,Tc,Dx,Cm,Co	21	NC	R
Ap,Ax,Tc,Dx,Cm,Co	30	NC	R*
Ap,Ax,Sm,Tc,Dx,Cm	119	HLY	R
Ap,Ax,Sm,Tc,Dx,Cm,Km	60	HLY	R*

Total number of drug resistant haemolytic strains = 17
Total number of drug sensitive haemolytic strains = 10
* Transferable in nature
NC - Non characterised

Table 41 : Correlation between haemagglutinating phenotypes and drug resistantce among 111 strains of Escherichia coli

Haemagglutinating strains		Percentage
Phenotypes	Total no. of resistant strains	
$MSHA^+ / MRHA^-$	47	42.34
$MSHA^+ / MRHA^+$	23	20.73
$MSHA^- / MRHA^+$	6	5.40
$MSHA^- / MRHA^-$	35	31.53

Total number of drug resistant haemagglutinating strains : 76 (68.48%)

DISCUSSION

The term 'pathogenicity' denotes the ability of a bacteria to cause disease. Pathogenicity is a taxonomical attribute being the property of a species. The individual strains of bacterial species may, however, vary widely in their ability to harm the host species and this relative pathogenicity is termed as virulence. Virulence is accordingly an attribute of a strain, not a species, one may speak of a highly virulent, a weakly virulent or even an avirulent strain. Highly virulent strains means, the accumulation/acquisition of various virulence factors in a single bacterial cell.

The molecular basis of plasmid induced pathogenicity of bacterial diseases is emerging as a new area of bacteriological research. Plasmids in *Escherichia coli* are responsible for causing outbreaks of gastroenteritis in human and animals. Many of *E.coli* virulence factors such as enterotoxins, colicins, haemolysins, adhering antigens and drug resistance are plasmid determined i.e. they are present on extra chromosomal genetic elements. Plasmids are often unstable and their spontaneous losses may occur, which alters the bacterial virulence. The central objective of this investigation is to study various plasmid encoded properties individually and their relationship with each other in various combinations by either transferring the specific virulence character to an avirulent recipient strain or by eliminating the particular plasmid (coding for virulence) by suitable curing agents, so that a better chemotherapeutic approach may be developed against such pathogens.

BIOCHEMICAL AND SEROLOGICAL STUDIES OF *ESCHERICHIA COLI* STRAINS:

One hundred and ninety four strains of *E.coli* isolated from human (UTI and GIT), animals (GIT) and poultry (Septicaemia) were identified

and characterized by using a total of 22 biochemical (11) and sugar fermentation reactions (11). Most of the strains demonstrated typical biochemical reactions, barring a few which showed some atypical reactions like indole negative by only four strains. Similar pattern of biochemical behaviours of *E.coli* strains were also reported by Yadava and Gupta (1969); Edward and Ewing (1972), and Rajani (1981).

Most of the sugars produced common reactions. Only a group of five sugars (dulcitol, sucrose, salicin, raffinose and adonitol) were fermented heterogeneously. These variable fermentation reactions are not only useful for differentiating the various *E.coli* strains from each other but also help to study the epidemiology and pathogenicity of the strains in relation to the disease they cause. Several authors have pointed out that dulcitol fermentation by *E.coli* strains is closely associated with the pathogenicity of the bacterium (Harry and Chubb, 1964; Yadava and Gupta, 1969). Some workers suggested that raffinose, sorbitol and arabinose fermentation might also be associated with pathogenicity of *E.coli* strains (Schmitt *et al.*, 1979). In the present study also fermentation of dulcitol, sucrose and raffinose may be correlated with virulence and pathogenicity of *E.coli* strains. Fermentation of some sugars like raffinose, lactose, sucrose have been shown to be mediated by plasmids. This advantage resides in the potential to utilise these sugars (present in vegetary foods) as source for the synthesis of capsular antigens like K88, K89 etc. These K antigens of *E.coli* containing galactose, glucose, N-acetyl glucosamine and colitose are know to interfere with the host defense by preventing phagocytosis. (Johnson *et al.*, 1976; Smith, 1977).

Serotyping is still commonly used to identify the pathogenic *E.coli* strains which is based on three different antigenic structure. These are

somatic (O), capsular (K) and flagellar (H) antigens. All the three antigens play an important role in the pathogenic strains. In the present study the serotyping was based on somatic (O) antigens due to non-availability of antisera for K and H antigens at the Typing Centre. Somatic serogroups (O9, O35, O60, O68, O70, O103 and O147) of animal origin were encountered most frequently among monkey isolates. Strains isolated from human sources belonged to serogroups O2, O4, O5, O15, O20, O25 and O143. WHO (1980) has described 17 'O' serogroups which are universally recognized as a cause of epidemics of infant diarrhoea in many countries. These groups are O18, O20, O25, O26, O28, O44, O55, O86, O111, O112, O114, O119, O125, O126, O127, O128 and O142. Similarly, most common 'O' sero groups of farm animals are O8, O141, O130, O149, and O157, (Wray *et. al.*, 1993). Some of the O-serogroups like O5, O12, O106, and O143 are generally considered as animal pathogens were also isolated from human beings in the present study. Similarly, at least five O-sero groups namely O20, O25, O44, O55 and O86 generally known to occur in human diarrhoea were presently isolated from monkey diarrhoea also. Ewing *et al.* (1955) and Schemneider, *et al.* (1960) have also isolated certain human serotypes (O26, O55 and O111, from diarrhoeal cases of non-human primates. Isolation of human O-serotypes O2, O6, O86, O126 and O128 was also reported from diarrhoeal cases of domestic animals by Yadava (1966). Rajani (1981) also reported occurrence of human 'O' serogroups (O18, O20, O25, O28, O44, O86, O126, O128) from farms animals showing acute diarrhoea.

The above findings thus, revealed the zoonotic importance where there is a free transmission of *E.coli* serotypes from man to animals and vice versa which may pose a serious veterinary as well as human health

problems. In the recent years it has become possible to made a fine distinction among pathogenic and non-pathogenic isolates of *E.coli* based on their several structures like capsule, cell wall, flagella and pili and products like enterotoxins, haemolysins, colicins, serum resistance and drug resistance. In the present study these characters were assessed individually and in combinations to characterize the pathogenic potential of *E.coli* strains under study .

PLASMID MEDIATED DRUG-RESISTANCE :

The acquisition of antimicrobial resistance provides a mechanism of survival in intimating environment to a bacterium which in turn is an impediment to bacterial chemotherapy. In present era, the multiple drug resistant strains are common in the random population screened. Such resistance is generally plasmid encoded or transposon mediated. These plasmids are free to mutate without lethal effects on the host cell. They can also move between cells and species even genera, accumulating gene by conjugation/recombination. The prevalence of multiple drug resistance in bacteria itself is a serious problem to chemotherapy. Transfer of R-factors to other members of the family Enterobacteriaceae specially to *E.coli, Salmonella* and *Shigella* etc. make it even of greater concern to cilinicians, particularly in curbing the diseases like typhoid, diarrhoea, dysentery and other gastro-intestinal and extra-intestinal infections both in human and veterinary practices.

In the present investigation, out of 194 isolates, 111 (57.21%) were found resistant to one or more drugs, of which 69% strains were multi-

resistant to three or more drugs simultaneously. Drug sensitivity studies carried out 27 years ago, in this laboratory from the same geographical region Lucknow, showed that the incidence of resistant strains among *E.coli* strains was only 13.8% (Yadava, 1966). There has been approximately five fold increase in the frequency of drug resistance during this period. Such increasing trend in the frequency of drug resistant isolates have also been reported by some workers specially in developing countries (Levy, 1982; Farrar, 1985; Mc Gowan, 1983 and 1987). The incidence of multiple drug resistance itself is an indication of the plasmid encoded resistance because host chromosome can not afford for such an extra-load of various drug resistance gene pool. Although, chromosomal mediated multiple drug-resistance have also been reported, but rarely (Hachler, *et al.*, 1991; Cohen, *et al.*, 1993).

In the present study a higher level of resistance (MIC > 1600 µg/ml) against ampicillin, amoxycillin, tetracyclinec, doxycycline, streptomycin, chloramphenicol and cotrimoxazole were recorded in many of the strains which is again an indication of the involvement of plasmids encoding such resistance. The ability of a strain to tolerate a drug concentration depends upon the efficacy of expression of the gene encoding for drug resistance. Some times, repetitive sequence of the nucleotide cluster encoding for biosynthesis of an enhanced amount of enzyme (gene dosage effect) may also be a cause of higher level of resistance. Plasmids are small replicon where expression is quite efficient, plasmid mediated drug resistance strains can, therefore, tolerate higher level of drug concentrations.

In the present investigation higher incidence of drug resistance against ß-lactam antibiotics (ampicillin and amoxycillin), tetracycline, doxycycline, streptomycin and chloramphenicol were observed more fre-

quently than other antibiotics. This might be due to the early use of these antibiotics in chemotherapy. Thus, the resistance to them has spread over a period of time giving rise to comparatively high incidence of resistant strains. The isolates resistant to co-trimoxazole, kanamycin and nitrofurantoin occurred less frequently, probably due to their recent introduction to chemotherapy. Varying frequencies of multiple drug resistance have been reported by various workers among *E.coli* strains of man and animal origin (Trishkina *et al.*, 1977; Diwan and Sharma, 1978; Agarwal *et al.*, 1984; Nandivada and Amyes, 1990; Singh *et al.*, 1992). The variation of drug resistance frequencies from study to study might be due to the differences in local ecology and pressure of drugs in a particular community as well as due to the different standards adopted for measuring the level of drug resistance (because no uniform standard for resistance level has yet been fixed).

In the present study resistance against 4- quinolones (nalidixic acid and norfloxacin) were not detected. The flouro-quinolones including norfloxacin, ciprofloxacin and ofloxacin are synthetic drugs have been introduced relatively recently and are not yet subject to horizontally transmitted positive function resistance. This may be due to the lack of plasmid mediated resistance against 4-quinolone in bacteria and thus signifies the importance of these drugs in chemotherapy. However, reports on emergence of chromosomal resistance against 4-quinolones among *Staphylococci* and *Pseudomonas* sp. have recently been demonstrated by many workers (Piddock and Wise, 1989; Courvalin, 1990; Lewine *et al.*, 1990; Ahmad and Akhtar, 1992).

Out of 111 drug resistant strains so obtained 35 (31.53%) strains possessed conjugative R-plasmids. Prevalence of Tra^+ R-plasmids was

more (46.53%) among human strains as compared to 25% among animal (monkeys) isolates. This finding is in agreement with some of the previous workers (Adetosy, 1980; Alsowagh and Shibl, 1981; Agarwal *et al.*, 1984). However, Young *et al.* (1986) and Lamikanra *et al.* (1990) have reported 60% and 54.27% incidence of transferable R-plasmids among *E.coli* strains. These figures are slightly higher as compared to our present study.

The emergence of transferable multiple drug resistant bacterial isolates from wild (monkey) and laboratory animals is of great concern because of their potential to exchange these resistance genes between pathogens of man and animals causing zoonotic problems. High transfer frequencies of resistance against Ax, Ap, Co, Cm, Tc reduced the significance of these drugs in chemotherapy.

Some plasmids which have partially lost their transfer functions, can be transferred with the help of transfer function taken from other transferable plasmids. Such plasmids have retained a region called the mobilization region (mob.). These non-conjugative plasmids are also clinically important because conjugative plasmids present in bacterial population can mobilize them easily to other organism. In this study 13.63% of 35 non-conjugative highly resistant strains of monkey origin mobilized their drug resistance markers in different combination with the help of mobilization factor *E.coli* X^+. Few other workers (Anderson, 1965; Nandivada and Amyes, 1990) have also done similar type of experiments by using mobilization factors.

Non-tansferable R-plasmids also stabilize the resistance in the bacterial population and put hindrance in chemotherapy. Such plasmids can be detected by performing curing studies. In the present investigation,

therefore, such plasmids were detected among 7 highly resistant strains of human origin by using one or more curing agents such as norfloxacin, acridine orange and sodium dodecyl sulphate (SDS) at a dose of sub minimal inhibitory concentration level. Our these findings are in agreement with other workers. (Hooper *et al.*, 1984; Wiesser and Wiedman, 1985 and 1986, Singh and Yadava, 1988).

By using all the three methods, (i.e. conjugation, mobilization and curing assay) 48 strains were detected to possess plasmid mediated drug resistance. No separate studies were conducted to find out the chromosomally mediated resistance but the resistance in all remaining strains can not be considered to be mediated by chromosome only because certain plasmids are refractory to transfer, mobilization and curing functions (Singh and Yadava, 1988).

PLASMID MEDIATED VIRULENCE PROPERTIES:

ENTEROTOXINS :

For the detection of enterotoxins production among *E.coli* strains, animal models (Rabbit ileal loop for LT and Guinea pig ileal loop for ST enterotoxins) were used. Although, it is laborious, expensive and need a good experience, but is considered to most reliable method. Guinea pig ileal loop model was developed in our laboratory. This method is very sensitive to both types of ST enterotoxins (STa and STb) while infant mouse model previously used for detection of ST enterotoxins is only sensitive to STa type of enterotoxins and can not detect STb enterotoxin. Further, more number of infant mice are needed to detect a single enterotoxigenic

strain. On the other hand by guinea pig ileal loop method, many strains could be screened in a single animal.

The study for production of enterotoxins among 194 strains of *E.coli* showed that about 51% of the strains were toxigenic. Majority (74%) of the ETEC strains were ST producers while LT and ST/LT producers were encountered almost equal (nearly, 13%). The high incidence (52%) of enterotoxigenic strains from poultry and human UTI (48.5%) isolates explains their clinical importance. It is possible that these ETEC strains have their ultimate source from gastro intestinal tract. Other workers have also reported high percentage in varying frequencies of enterotoxigenicity among *E.coli* strains. (Goldhar *et al.*, 1980; Saxena and Yadava 1985 and 1986; Thomas *et al.*, 1987; Broes *et al.*, 1988). Reports on the prevalence of ETEC strains from monkey diarrhoea are not available from India. Our study shows that an appreciable number (60.9%) of monkey isolates produce enterotoxins (ST, 73%; LT, 17.3% and LT/ST, 9.6%) which indicates that the heat stable enterotoxins play an important role in monkey diarrhoea.

The nature of enterotoxin gene have been found to be located both on transferable or non-self transferable plasmids (Ent-plasmids) by several workers (Smith and Linggood, 1970; Gyles *et al.*, 1974; Wacshmuth *et al.*, 1976; Singh *et al.*, 1992; Bertin, 1992). In the present study about 13% of ETEC strains transferred their enterotoxins character into non-enterotoxigenic recipient strain (*E.coli* K-12) by single step bacterial cross. Since Ent$^+$ cells (ETEC) have no phenotypic different characters which can be used for direct selection of Ent$^+$ *E.coli* from mixed population, therefore, antibiotic resistance markers were chosen to select transconjugants which accepted Ent-factors. It was assumed in this study that Ent$^+$ strains which could not

transfer their Ent-factors by conjugation might have possessed non-transferable plasmids.

COLICINS :

Colicins mediated by Col-plasmids provide bacteria the means of self establishment against a competitive struggle for existence in the environment of intestinal tract of man and animals by eradicating colicin sensitive microflora. By now, it has become evident that colicin production is also related to increased virulence and pathogenicity of the bacterium (Waters and Crosa, 1991). The strains possessing Col-V plasmid have been reported to be very pathogenic. Binns *et al.* (1979) found that elimination of Colicin-V plasmid from human bovine, ovine and avian strains invariably reduced their pathogenicity in experimental animals. In 1980, Goel and co-workers found a close association of various Col-plasmids with the pathogenicity of *E.coli* strains. They also reported the establishment, long survival and multiplication of colicinogenic strains in animal intestine. Thus, enhancing the virulence and pathogenicity of colicinogenic strains.

In the present investigation, a low (11.8%) incidence of colicinogenic *E.coli* strains was recorded from man and animals. Similar findings have also been reported by many other workers (Njoku-Obi *et al.*,1978; Ansari and Yadava, 1981; On the other hand some workers have also reported a high incidences (30-48%) of colicin production among *E.coli* strains (Schal, 1974; Obi and Bardley, 1991).

High incidences (51.5%) of colicin factors transfer to recipient strain were observed during the course of present investigation such high transferability of Col-factor has also been reported by other workers

(Smith, 1974 and Riley and Gordon, 1992). These transferable Col-plasmids can spread from an antibiotic sensitive strains to a resistant strains in vivo providing quantum leaps towards virulence.

It has been recorded in the present study that colicin production in some strians are also associated with R-plasmids, haemolysins, adhering antigens and enterotoxins production. Therefore, transmissibility of colicin plasmids may help in dispersal of these virulence genes within a host adopted bacterial population, thus, molecular linkage of some of above virulence genes with colicin gene may provide an increased adaptive advantage.

HAEMOLYSINS :

An important property contributing to the pathogenicity of many bacterial species including *E.coli* is to produce haemolysins. Out of 194 strains only 27 (14%) strains produced haemolysin either of α or ß types. Other workers (Smith, 1963; Short and Kurtz 1971; DeBoy, 1980; 1983; Ahmad and yadava, 1980) have also reported haemolysin production among *E.coli* strains in varying frequencies. Maximum number (40%) of haemolytic strains was detected among human UTI isolates followed by GIT isolates of human and monkey (12.12% and 10.3% respectively). High incidences of haemolysin production among UTI isolates of *E.coli* have also been reported by Hughes *et al.* (1983); Garcia *et al.* (1985); Yadava *et al.* (1985); and Fule *et al.* (1990)

In this study few haemolytic strains, causing profused, bloody, fatal diarrhoea, were isolated from monkeys suggesting involvement of haemolysins. The exact role of haemolysin in diarrhoeal diseases is not yet clear. However α-haemolysin producing strains have clear advantage when

compared with non-haemolytic strains for colonization in pig intestine (Hinton *et al.*, 1985; Hampson *et al.* 1986). Besides this, Gillard *et al.* (1989) and Beutin *et al.* (1990 b) have reported that haemolysin contributes as a virulence factor in enteric infections of new born infants due to P-fimbriated *E.coli*. It has also been reported that α-haemolysin interferes with phagocytosis and has a toxic effect on phagocytes which indicates that it plays an important role in *E.coli* infection by counter acting a major host defence system (Bhakdi *et al.*, 1989; Gadeberg *et al.*, 1989).

In an attempt present study only 3 haemolytic strains could transfer their haemolytic character (HLY-plasmids) to one or other recipient strains (*E.coli* K12, *E.coli* PB-176 and *Salmonella typhimurium*) in the present study. Smith and Halls 1967; Noegel *et al.*, 1982 and Prada *et al.*, 1991 also demonstrated transferable nature of Hly-plasmids. Interestingly, the haemolysin factors were cured from 4 strains by using norfloxacin, acridine orange and SDS as the curing agents. Mitchell and Kenworthy (1977) also used curing agents to eliminate successfully the Hly-plasmids from host bacteria.

The haemolytic factors could not be transferred/ cured from majority (70%) of the haemolytic strains tested. It is possible that in such strains genetic determinants for haemolysin might be located on chromosome instead of plasmids. Similar observations were also made by Minshew *et al.* (1978), Hull *et al.* (1982); Welch and Falkow (1984) and Falbo (1992).

Further, the role of haemolysins on virulence and pathogenicity of bacterium was studied by mice mortality test, inoculating 0.17×10^8 CFU of bacteria per mouse in different batches of mice. It was observed that mortality caused by haemolytic *E.coli* strains of UTI origin was high as

compared to strains from GIT, which signifies an important role of haemolysins in urinary tract infections.

Effect of Haemolysins on mortality of Swiss mice:

In order to elucidate the effect of haemolysin production on the virulence of organisms under study, curing of HLY-factors from parent strains was under taken. It was observed that the curing of HLY-factors from the parent bacterial host led to the great reduction of the mice mortality percent in its HLY-derivative homogenic strain. This reduction in the percentage mortality, too, varied from one strain to another strain tested.

Similarly, the influence of HLY-factors on the virulence and pathogenicity of bacteria was further evaluated by transferring this factor as such to a non haemolytic *E.coli* K-12 recipient strain and examining then the mortality patterns of Swiss mice. No significance difference in the mortality patterns of such HLY-plasmids bearing recipient strain was observed, as it was either equal to or slightly less than that of their respective donor strain.

These findings are in agreements with the reports of Welch and Falkow (1984) who have also observed variation in the degree of virulence of haemolysin plasmid carrying *E.coli*. This variation in virulence by recipient strain might be due to some variation in the nucleotide sequence of the structural gene (Hly-A) for haemolysin (Berger *et al.*, 1982) Our findings have therefore, revealed clearly that haemolysin production may not be the sole but may be an important virulence attribute among *E.coli strains* causing intestinal and extra-intestinal infections.

ADHERING FACTORS :

Bacterial adherence to epithelial cells by virtue of pili or fimbriae is a recognized virulence factor, and it facilitates colonization of the micro-organisms in the upper intestinal tract, urethra and urinary bladder. These plasmids/chromosomal mediated fimbriae also attach to erythrocytes causing observable haemagglutination. The agglutination of erythrocytes of various species by bacteria is an indirect evidence of adhering factors being present (Finlay and Falkow, 1989).

Haemagglutination may be mannose sensitive (MS) or mannose resistant (MR). The precise contribution of individual fimbrial adhesins to bacterial pathogenicity has been difficult to define, because a single *E.coli* strain can express several distinct types of fimbriae or adhesins encoded by plasmid and/or chromosome. This genetic diversity permits an organism to adopt to its changing environment and exploit new opportunities presented by different host surfaces.

Out of 194 *E.coli* strains 150 (77.32%) showed either mannose sensitive haemagglutination (MSHA) or mannose resistant haemagglutination (MRHA) or both (MSHA/MRHA) with one or more erythrocytes of human type A^+, guinea pig, fowl, rabbit, sheep and bovine. Different patterns of MSHA and MRHA were observed. Similar observation was also reported by Girardean *et al.* (1988). Majority of the strains were positive for MSHA with guinea pig erythrocytes indicating the presence of type-I fimbriae while different patterns observed with other strains might be due to the presence of different fimbrial adhesins. Although several workers (Otto, 1975; Duguid and Old, 1980; Reid and Sobel, 1987) have reported the specific role of type-1 fimbriae on *E.coli* surface for colonisation of large bowl, urinary tract, urethra and mucosal structure but the exact role of

type-1 fimbriae in pathogenesis of diarrhoeal diseases is not well understood.

Mannose resistant haemagglutination (MRHA) is also an important virulence factors associated with virulence of bacterium of diarrhoeal and extra-intestinal infections. (Orskov, 1985; Blanco *et al.*, 1990 and 1991; Nihal *et al.*, 1992).

In the present study MRHA were detected against 64 strains of *E.coli* and maximum haemagglutination was observed against human type A^+ erythrocytes. Different patterns of MRHA were recorded which might be due to the presence of various fimbrial adhesins of *E.coli* strains (Gasstra and de Graaf, 1982; Saxena and Yadava, 1985; Oudega and Graff, 1988; Moon, 1990).

E.coli strains, not showing any haemagglutination with any erythrocytes might be having other fimbrial adhesins which did not cross react with erythrocytes used in the present study, e.g., fimbrial antigen 987 of porcine origin could not agglutinate with above erythrocytes (Gaastra and de Graaf, 1982). It is also possible that some strains might be having different adherence mechanism which could not be detected by MSHA or MRHA e.g. Hep-2 and Hela-cell adherence factors of EPEC strains (Pal and Ghose, 1990; Casey *et al.*, 1992. In some strains it is also possible that strains have adhesin antigen but it could not be detected in present study because the expression of some fimbrial antigens in vitro is affected by types of media, pH, and temperature of incubation as have been reported by Gaastra and de Graaf (1982) and Gally *et al.* (1993). In our conjugation experiments plasmid mediated transfer of adhering factors was detected among 16.66% of UTI strains by conjugation with *E.coli* K-12 indicating

implication of plasmids encoding such virulence determinants.

INTER-RELATIONSHIP BETWEEN VARIOUS VIRULENCE FACTORS OF *E. COLI* STRAINS :

Bacterial virulence is a consequence of multiple genetic loci. Even in infectious diseases associated with a single dominant virulence factor such as cholera toxin, the process of pathogenicity requires a number of multiple regulatory and structural gene elements. The epidemiology of enterotoxigenic *Escherichia coli* is not simple as it needs further explanation of inter-relationship between enterotoxins, adhering factors, and other potential virulence factors like colicin, haemolysin and serum resistance.

ASSOCIATION OF ENTEROTOXIGENICITY AND ADHERING PROPERTY OF *E.COLI*:

A strong correlation was observed between enterotoxin production and presence of adhering factors among *E.coli* population under study. Nearly 79% of ETEC strains showed various patterns of haemagglutination ($MSHA^+/MRHA^+$ & $MRHA^+$) with various erythrocytes in different combinations, which clearly indicated the presence of various types of adhering factors among ETEC strains. It was further interesting to note that ST, LT and ST/LT enterotoxins production was associated with different haemagglutinating phenotypes at different frequencies. Similar findings have also been reported by other workers (Gaastra and de Graaf, 1982; Saxena and Yadava, 1985).

A close correlation was also noticed between ST and LT/ST production with MHRA which was detected in 31% and 33% of ETEC strains respectively, while only 22% of LT producing strains showed MRHA reactions. Among human ETEC strains only 35% could show MRHA

reaction with human type-A erythrocytes which indicated the presence of colonization factor antigen (CFA-I). These results are in agreement with the reports of earlier workers (Evans *et al.*, 1977 and 1978; Lopez-vidal *et al.*, 1990).

CORRELATION BETWEEN THE PRODUCTION OF HAEMOLYSINS, ADHERING FACTORS AND ENTEROTOXINS :

A strong correlation was found between haemolysin production and presence of the adhering factors among the test isolates. Nearly, 82% of the haemolytic strains showed presence adhering factors (MRHA types). Low and coworkers (1984) showed that the gene encoding haemolysin production and MRHA were closely linked in *E.coli* strains of UTI origin. These authors also suggested that linkage of these two factors occurred through a transposition event and conferred a selective advantage to the isolates involved. Some other workers have also reported a similar close association between haemolysin and MRHA (Evans *et al.*, 1981; High *et al.*, 1988; Hacker 1989; Johnson, 1991).

Production of haemolysin and ST enterotoxins simultaneously was recorded among 41.4% of UTI isolates. Although, the role of enterotoxins in UTI infection is not defined, Prada *et al.* (1991) reported that plasmid encoded haemolysin production has a strong correlation with ST toxin production among *E.coli* strains of canine origin.

Our results emphasis that Hly-plasmids and ST plasmids may be having a strong compatibility or affinity in *E.coli* isolates particularly of UTI origin.

VIRULENCE FACTORS AND DRUG RESISTANCE PLASMIDS: A CORRELATION

Transmission of plasmid encoded virulence determinants among

bacterial population by plasmids and transposons are widely prevalent. Various reports concerning to the transmission of such genes from person to person (Linton, 1972), animal to animal and animal to human beings (Levey *et al.*, 1976; Linton, 1977 and 1986) and human to animal (Rolland *et al.*, 1985) are available in literature.

Heterogenic transfer by conjugation, under natural condition from gram -ve to gram +ve bacteria and vice versa (Trieu-Cuot *et al.*, 1987 and 1988) and even inter-kingdom transfer i.e. from bacteria to yeast and plant cell, has also been reported in the recent years (Stachel and Zambriski, 1989; Heinemann, 1991). These findings indicate that conjugation is a non-specific transfer process. The pathogenic *E.coli* strains harbouring drug resistance gene as well as other virulence determinants (enterotoxins, colicins, adhering factors) on the same plasmids or on separate plasmids could then allow further evolution of pathogenic potential of the bacterium and their co-transferability may be of great advantage for the survival and colonization of bacterial pathogens against the intimidating environment.

Enterotoxin versus drug resistance:

A strong correlation was observed with Ent-factors and antibiotic resistance factors among many of our *E.coli* strains. About 58.58% of enterotoxigenic strains of *E.coli* were found resistant to one or other drugs in various combinations. Nearly 65% of ETEC strains were multi-resistant to three or more drugs. The reports of Goldhar *et al.* (1980) , Choudhary *et al.* (1988), Lamikanra *et al* . (1990) also demonstrated a close association of drug resistance with enterotoxigenicity among *E.coli* strains of different clinical origin.

A close affinity of R-plasmids to enterotoxigenicity was observed

in this study as evident by a very high frequency (54.16%) of co-transfer of R-plasmids and enterotoxigenicity among 58 R^+ ETEC strains. It showed that gene responsible for enterotoxins biosynthesis might be present on plasmids which have strong affinity to co-exist or to recombine with transferable R-plasmids. Simultaneous occurrence of enterotoxins and drug resistance determinants among ETEC strains and occasionally on same plasmid have also been reported by many workers (Gyles *et al.*,1977; Echeverria and Murphy, 1978; Steglitz, *et al.*, 1980; Singh and Yadava, 1992).

As regards zoonoses, simultaneous transfer of R-plasmids and Ent-factors among *E.coli* isolates from monkey diarrhoea is of great zoonotic importance. Isolates bearing such plasmids are likely escape from animal body into environment such as water or sewage or may come in direct contact to monkey handlers. Such strains may transfer their plasmid to either other human and or animal pathogens, which in turn may infect human through some of their host serotypes which are of zoonotic nature. Antibiotic selection may also facilitates formation and translocation of such virulent R-plasmids among bacterial flora.

Colicins Production versus drug resistance:

Another virulence factor associated with intestinal and extra-intestinal *E.coli* infections is colicin which is also said to be mediated by transferable or non-transferable plasmids (Col-plasmids).

In the present investigation, 57.57% of colicinogenic strains were found resistant to one or more of the drugs tested. The plasmids encoded drug resistance was detected among 36.48% of Col^+R^+ strains. Interestingly R-plasmids and Col-factors could be transferred simultaneously among 4

colicinogenic strains. Our study reflects that Col-factors and drug resistance plasmids have a strong affinity to co-exist and therefore, transferred simultaneously. Such co-rrelation has been reported by few other workers also (Goel and Yadava, 1985; Singh *et al.* , 1989). The spread and formation of such drug resistant colicinogenic plasmids will be an adaptive advantage to the bacterium under adverse conditions (high drug pressure and competition for colonization) and may also provide increased virulence property to the drug resistant bacterial cells. The Col-plasmid (Col.Ib) was found to encode the colonisation factor antigen as reported by Williams *et al.* (1978). It is possible that some of the Col^+ R^+ $MRHA^+$ strains of the present study may also have such properties located on single plasmid or on plasmids of strong compatibility. Presumably, recombination of R and Col-CFA plasmids may take place and eventual introduction of Ent-plasmid in such bacteria could then allow further evolution of the pathogenic potential.

Haemolysin Vs. drug resistance :

Though incidence of haemolysin production was insignificant among present bacterial population, majority (62%) of haemolytic strains were resistant to one or more drugs tested. Among these one strain transferred its haemolytic character and Kanamycin resistance simultaneously and four other strains showed elimination of HLY factors and drug resistance simultaneously by one or other curing agents employed.

Strains harbouring both HLY and R-factors on separate plasmids or possibly on same plasmid may create very serious problem in curbing extra-intestinal infections. Although, transfer of haemolytic factors is not very common, therefore many workers (Bhalla and Agarwal, 1989; Fule *et al.*, 1990) could not demonstrate haemolysin transfer among drug resistant

strains of UTI origin.

Haemagglutinating properties (Adhering factors) Vs. drug resistance :

Various pathogenic bacteria including *E.coli* require various adhering factors for their attachment and colonization in host cells. Most of the *E.coli* strains isolated from intestinal and extra-intestinal infections harbour various types of fimbrial adhesins. Many of these fimbrial adhesins are plasmid encoded (K88, K89, CFAII, CFAIV, etc.). In the recent years some workers have reported the occurrences and transfer of adherence properties and antibiotic resistance simultaneously in *E.coli* and *Klebsiella pneumonia* (Laporta *et al.*, 1986 and Michaud *et al.*, 1992). In the present investigation an appreciable number (68%) of *E.coli* strains having different patterns of either MSHA or MRHA haemagglutination were found to be resistant to one or more drugs. Out of 48 R-plasmid harbouring *E.coli* strains so obtained, majority (68.18%) of them harboured different adhesins as indicated by their different haemagglutination patterns.

This study shows that nature and outcome of an *E.coli* infection does not depend upon a single virulence factor alone but it is a multifactorial phenomenon. The virulence and pathogenicity of an organism is directly proportional to the number and nature of virulence factors accumulated in it.

All of our results reflect that *E.coli* strains harbouring various plasmid encoded virulence factors have close correlation with drug resistance plasmids, which are widely present among gram -ve bacteria. The presence of such plasmids, although have been biologically indicated, these could have been physically viewed in a better way by performing suitable electrophoretic experiments on isolated bacterial DNA as abun-

dantly reported by a number of workers (Gyles *et al.*, 1974; and 1977; Wachsmuth *et al.*, 1976, Echeverria and Murphy, 1978; Silva, 1983; Danbara *et al.*, 1988; Singh and John, 1991; Singh *et al.*, 1992). Genetic location of virulence genes and drug resistance genes are mostly found on closely located plasmids and in many strains, probably on the same plasmids. It is expected that this type of genetic combination may appear more frequently in future due to the powerful selection pressure created by the extensive use of antibiotics particularly in developing countries like India. In such countries where sanitary facilities are very primitive and antibiotics are used indiscriminately, this condition may create more serious chemotherapeutic problems.

Transferable nature of plasmids and wide spread occurrence of transposons both on bacterial plasmids and chromosome will further increase the accumulation, formation and spread of multi-virulence plasmids. Accumulation of such different virulence factors (probably due to their strong affinity to each other) will increase the virulence many folds. Such strains will have an extra advantage to the isolates involve in intestinal and extra-intestinal infections of man and animals. Because of their free transmission from man to animals and vice-versa, they may also create very serious zoonotic problems.

SUMMARY

Pathogenic bacteria have a chemical armoury which enables them to invade a host and produce diseases. The problem is to identify the weapons in this armoury, their relative importance, chemical nature and mode of action on the host. This task is relatively simple when pathogenicity is determined by a single bacterial product, easily produced *in vitro*, as in diphtheria and tetanus. However, in majority including *Escherichia coli* infections the pathogenicity can not be related to a single microbial product and its biochemical basis are difficult to identify.

A significant development in our understanding of *E.coli* infections took place when it was discovered that the genetic determinants of certain virulence characteristics such as enterotoxins and adhesiveness could be carried by plasmids. However, the presence of plasmids is not always required for virulence nor it is the only factor involved.

Plasmids are sometime unstable and this may make it possible to relate changes in the virulence of the organism with the loss of plasmids. Re-introduction of the plasmid into the wild type and indicator recipient strains can then confirm that genes coding for a particular character carried on the plasmid.

Except for enterotoxins however, little is known about the precise role and relative importance of these virulence factors in determining the pathological potential of bacteria in the development of pathogenic conditions.

The present investigation was conducted to explore the presence of various plasmid encoded characters including drug resistance and various virulence factors (enterotoxins, colicins, haemolysins and adhering factors), their infectious nature and role in influencing the virulence

and pathogenicity of *E. coli* strains. These virulence factors have also been investigated for their affinity with drug-resistance plasmids in various combinations under natural condition. The results and conclusion drawn in the present investigation can broadly be divided under following heads.

BIOCHEMICAL AND SEROLOGICAL STUDIES:

A total of 194 strains of *E.coli* were isolated from human (UTI,30 strains; GIT, 33), monkeys (GIT, 97), poultry (Septicaemia, 24) and rabbits (GIT, 10). All strains were identified as *E. coli* on the basis of their cultural, morphological, biochemical and sugar fermentation reactions. Twenty two tests were performed (11, biochemical and 11, sugar fermentation reactions) to characterize and differentiating the strains from each other. One strain was found utilizing citrate as sole source of carbon and four strains were negative for indole test. More than 95% strains fermented lactose, arabinose, xylose, sorbitol and rhamnose while adonitol and inositol were fermented only by 5.6% and 1.0% strains respectively. Other sugars e.g. dulcitol, sucrose and salicin were fermented by 43% to 66% of the strains. On the basis of heterogenous fermentation of 5 selected sugars (i.e.. dulcitol, sucrose, salicin, raffinose and adonitol), 194 *E.coli* strains were grouped into 24 biotypes. The fermentation of dulcitol, sucrose and raffinose, were correlated for their association with virulence and pathogenicity of the *E. coli* strains, like pathogenic 'O' serogroups.

The serotyping of 194 strains of *E. coli* was carried out at National *Escherichia coli* Typing Centre Kasauli, (H.P.), India against available 'O' antisera. Out of 194 strains, 112 were typed and distributed among 54 'O' serogroups. Maximum number of 6 strains were recorded in two serogroups

(O35 and O9) followed by serogroups O2, O25 (5 strains each); O25, O60, O68, O103 and O147 (four strains each); O1, O61, O73 and O84 (3 strains each) O15, O17, O22, O23, O38, O45, O70, O88, O93, O98, O143, and O159 (two strains each) and rest 28 'O' serogroups had only one strain in each group.

Out of 17 known human pathogenic 'O' serogroups (as described by WHO, 1980), five (O20, O25, O44, O55 and O86) were encountered among test strains isolated from non-human primates. Similarly, known animal pathogenic O-serogroups (O5, O12, O106 and O143) were also recorded in human isolates in the present study.

These serological finding clearly, indicate free transmission of pathogenic *E. coli* strains from human to animals and vice versa, thus, such strains are of great importance in term of bacterial zoonoses.

In addition to the above known pathogenic groups of man and animals, five other human pathogenic O-serogroups (O1, O2, O15, O158 and O159) were also encountered in monkey diarrhoea which were generally not known to be pathogenic in non-human primates. Although all these strains were isolated from frank diseased conditions. Therefore, the present criteria based on biochemical and serological classification alone is not sufficient to differentiate a strain to be pathogenic or non-pathogenic. Thus, the emphasis was given on the presence of some other virulence factors among *E.coli* population and their association with each other.

PLASMID ENCODED DRUG RESISTANCE IN *ESCHERICHIA COLI* POPULATION :

Antibiotic sensitivity behaviour and their level of resistance (Minimum Inhibitory Concentration, MIC) have been determined against 11 drugs namely ampicillin (Ap), amoxycillin (Ax), tetracycline (Tc), doxycycline (Dx), streptomycin (Sm), chloramphenicol (Cm), Kanamycin (Km), co-trimoxazole (Co), nitrofurantoin (Fd), nalidixic acid (Nal) and norfloxacin (Nr). In this investigation, 56.7% strains were observed to be resistant to various drugs simultaneously in different combinations (ranging from one to eight drugs), out of which 69% strains were multi-resistant to three or more drugs. The over all incidence of drug-resistance was maximum for Tc (41.23%) followed by Dx, Sm, Ap, Cm, Ax, Co and least for Fd and Km (nearly 3.5% each). None of the strain was found resistant to nalidixic acid and norfloxacin which signify the clinical importance of these drugs in human and veterinary chemotherapy. Incidence of drug resistance was more (69%) among human strains as compared to animal isolates (54%).

Resistant strains showed a wide range of drug resistance level. MIC values ranged from 12.5 to 3200 ug/ml for various antibacterial drugs. High levels of drug resistance in a particular bacterial strain is an apparent indication of involvement of plasmid DNA, mediating such resistance.

All 111 drug resistant strains so obtained were tested for conjugal transfer of their R-plasmids. About 31.5% of resistant strains transferred their drug resistance either partially or completely to the recipient *E.coli* K-12 strain. A representative group of 35 drug resistant non-conjugable strains were further tested for mobilization of their drug resistance determinants with the help of mobilization factor X^+ of *E. coli* and a repre-

sentative group of 19 drug resistant (non-conjugable and non-mobilizable) strains were also studied for plasmid elimination assay using three curing agents namely norfloxacin, acridine orange and SDS. Seven strains mobilized their R-factors and 6 strains were made to cure their R factors by one or more of the curing agents. The data indicated that norfloxacin was better curing agent as compared to SDS and acridine orange for eliminating R-plasmids of *E. coli*. Thus, a total of 48 drug resistant strains were demonstrated to carry R-plasmids (transferable and non-transferable).

PLASMID ENCODED VIRULENCE FACTORS OF *ESCHERICHIA COLI*:

ENTEROTOXINS:

Enterotoxins of *E. coli* are supposed to be the cause of acute diarrhoea both in infants of human and animals. Out of 194 strains of *E. coli*, 99 (51%) producing enterotoxins. Majority (74%) of ETEC strains were ST produced while LT and ST/LT producers were encountered almost equal (13% each). The occurrence of enterotoxigenic strains among poultry was 52% and in human UTI, 48.48%. Occurrence of such toxigenic strains in UTI may have dangerous implications because by contaminating and colonizing the bowl, they will lead to diarrhoea.

An interesting observation was that the ST enterotoxins played major role in causing acute diarrhoea in animals including monkey as was evident by their high incidence in animal ETEC strains. Auto-transferable nature of enterotoxin plasmids (Ent-factors) was detected among 22.41% of 58 R^+ ETEC strains. R^-ETEC strains could not be studied for the nature

of transferability of their Ent-factor due to absence of any direct selection markers.

COLICINS:

Another important virulence factors of *E. coli* strains which are associated with intestinal and extra- intestinal infections is said to be colicin production. Some colicin plasmids (colicin V, B & M) harbouring strains are more pathogenic and virulent in nature. In the present study, colicin production was detected among 33 (17.52%) of the strains using *E. coli* B, *E. coli* Row and *E. coli* K-12 as colicin sensitive indicator strains. It was observed that *E. coli* Row was the most suitable strain for colicin detection. Inter-generic transfer of Col-plasmids to other pathogenic bacteria like *Salmonella typhimurium* was demonstrated. Unlike *E. coli* K-12, which is colicin sensitive, *Sal. typhimurium* was found to be resistant against colicins of all 33 strains. Therefore for colicin transfer studies *Sal. typhimurium* was used as recipient. It was also found that transferability of Col-plasmid was as common as transfer of R-plasmid.

HAEMOLYSINS:

To explore the probable role of haemolysins on the pathogenicity of *E. coli*, we tested all 194 strains on 6% defibrinated sheep blood agar plates. Out of these, only 27 (14%) strains produced haemolysins. Maximum occurrence (40%) of haemolytic strains was recorded among human UTI isolates, followed by GIT isolates of human (12.12%) and monkey (10.3%). Haemolysin transfer study showed that only 4 of 28 (27 + 1 standard = 28) haemolytic strains could transfer their HLY factors to one or other recipients (*E. coli* K12, *E. coli* PB-176 and *Salmonella typhimurium*).

In our efforts to cure the HLY-factors, we could get very limited

success. Out of 24 non-transferable haemolytic strains only 4 could be cured for their HLY-factors by treating with 3 curing agents (norfloxacin, acridine orange and SDS). Low percentage of transfer or curing of HLY-factors in majority of these strains may be due to the presence of haemolysin determinants on chromosome rather than on plasmid.

EFFECT OF HLY-FACTORS ON VIRULENCE AND PATHOGENI - CITY OF *E. COLI*:

To access the effect of haemolysin on pathogenicity and virulence of its host bacterium, 27 haemolytic *E. coli* strains were tested in Swiss mice. All strains were inoculated intraperitoneally (i/p) at the dose of $0.17x10^8$ C F U /mouse and observed for mice mortality patterns upto 7 days. Different strains gave different patterns of mortality in mice. On the basis of their mortality patterns, haemolytic strains were divided into three groups as virulent (70-100% mortality), partially virulent (69-20% mortality) and avirulent (< 20% mortality).

After transfer of haemolysin factors, 2 conjugants of K-12 were further tested for their virulence in mice along with non-haemolytic isogenic recipient parent (*E. coli* K-12) and their respective donor (Hly^+ strains). All haemolytic K-12 conjugants showed mortality in mice while non-haemolytic parent K-12 caused no mortality. The total mortality percentage of transconjugants (Hly^+) was however less than their donor strains. Moreover, four parent haemolytic *E. coli* strains (*E. coli* Hly^+) and after, their subsequent curing of HLY factors (*E. coli* Hly^-) were also examined similarly in mice models for any change in their toxicity behaviour. Non-haemolytic derivative of parent strains showed less mortality in mice than their homogenic haemolytic parent strains. These findings indicate that haemolytic factor to be pathogenic and enhances the virulence of its

host bacteria. However, HLY factor alone was not decisive for virulence but additive with other factors because some parent haemolytic strains were found to cause very low mortality or very less reduction in mortality after eliminating HLY- factors.

ADHERING FACTORS:

Bacterial adherence to epithelial cells by virtue of pili or fimbriae is a recognized virulence factor, which facilitates colonisation of the bacterium in the upper intestinal tract, urethra and urinary bladder etc. of human beings and animals. These plasmid mediated fimbriae also attach to erythrocytes causing observable haemagglutination. This agglutination of erythrocytes of various species by bacteria is an indirect evidence of adhering factors being present. Out of 194 test strains, 150 (77.32%) showed either mannose sensitive haemagglutination (MSHA) or mannose resistant haemagglutination (MRHA) or both, with the erythrocytes of human type A+, guinea pig, fowl, rabbit, sheep and bovine. The strains thus, were grouped into different haemagglutinating patterns depending upon the type of fimbriae or adhesins present on *E. coli* surface. The strains expressing mannose sensitive agglutination with guinea pig erythrocytes provided evidence for the presence of common type -1 pili which may have their role in colonization of urinary tract, vaginal mucosa, urinary bladder and the large bowl.

A total of 64 (42.6%) strains of *E. coli* tested positive for MRHA reactions with the erythrocytes of one or more species under study. There MRHA adhesins may have a crucial role in pathogenesis of *E. coli* infections. Human strains showing MRHA with human type A^+ blood provided evidence for the presence of colonization factor antigen -1 (CFA-I) while some of the human isolates showed MRHA with bovine and sheep eryth-

rocytes which may be due to the presence of CFA-II. The presence of various haemagglutination patterns (MSHA/MRHA) among animal isolates may be due to the presence of different plasmid encoded adhesins on bacterial surface. These adhesins could be transferred to non-adhesin bearing recipient strain by conjugation. We found that a considerable number of UTI strains, (23.8%) transferred their haemagglutinating character(s) to *E. coli* K-12 recipient.

CORRELATION STUDIES OF VARIOUS VIRULENCE FACTORS:

Strong relationship between enterotoxigenicity and adhering factors, was observed among ETEC strains. Nearly 79% of ETEC strains showed various haemagglutinating phenotypes as $MSHA^+$, $MRHA^+$ and $MSHA^+/MRHA^+$. Not only this a close correlation was observed between ST and LT/ST production with MRHA phenotypes which was detected in 31% and 33% of ETEC strains respectively whereas only 22% of LT producing *E. coli* strains showed MRHA reactions. Among human ETEC strains 35% could show MRHA reaction with human type A^+ erythrocytes which indicated the presence of CFA-I. Only two enterotoxigenic strains of human origin exhibited MRHA with sheep/bovine erythrocytes.

High affinity was observed between haemolysins and adhering factors. Nearly, 81.48% of the haemolytic strains were found to possess mannose resistant haemagglutinins. Correlation also existed between haemolysin production and enterotoxin production. About 41.4% of haemolytic strains of human UTI origin produced heat stable (ST) enterotoxin. Although, the role of enterotoxins in UTI cases has not been defined, it has been suggested that HLY-plasmids and ST plasmids might be having strong compatibility or affinity to recombine in *E. coli* isolates of UTI origin.

ASSOCIATION OF VIRULENCE FACTORS AND DRUG RESISTANCE :

In the present investigation enterotoxin production and drug- resistance was simultaneously recorded in 59% of ETEC strains, showing a close correlation between the two. This was found to be in higher in human ETEC strains than in animals ETEC strains. A total of 65% of R^+ ETEC strains were found multi-resistant to three or more drugs. Very high incidence (54.16%) of co-transfer of Ent-factors and R-factors was also observed. It showed that gene responsible for enterotoxins biosynthesis might be present on plasmids which have strong affinity to co-exist or to recombine with transferable R-plasmids. Such diarrhoeal isolates from monkeys, therefore, are of great zoonotic importance.

Further, production of colicin and its association with drug-resistance have been analysed. It was observed that like enterotoxins, colicins also have a strong affinity with drug resistance plasmids. About 57.57% of Col^+ strains were resistant to one or more of the drugs. Co-transfer of antibiotic resistance and colicin production was also demonstrated in at least four strains. The formation and spread of such genetic combination will be an adaptive advantage to the bacterium. They will successfully compete, survive, multiply and colonise in the gut and other tissues even in the presence of antibiotics.

In this study a high affinity was observed between drug - resistance and haemolysin production. Majority (62%) of the haemolytic strains were found resistant to one or more drugs at a time. Although, co-transfer of haemolysin factor and drug resistance was found in one strain. During

plasmid elimination studies, HLY-factors and drug resistance markers were eliminated simultaneously from four *E. coli* strains. These results showed that at least in above five haemolytic strains genetic markers for haemolysin and drug resistance are located on plasmids having strong affinity to co-exist in the host bacterium.

In the present investigation an appreciable percentage (68%) of *E. coli* strains having different patterns of haemagglutination (MSHA or MRHA) were also resistant to one or more antibacterial drugs. This showed that like all other virulence factors adhering factors also have strong correlation with drug resistance plasmids in *E. coli* strains.

The present study revealed that plasmids play a crucial role in the virulence and pathogenesis of *E. coli* strains and the that nature and outcome of an *E. coli* infection does not depend upon a single virulence factor alone but it is a multifactorial phenomenon. It has also been established that plasmid encoded virulence factors of *E. coli* strains have close affinity (compatibility) with drug resistance plasmids, which are widely present among Gram -ve bacteria and can easily be transferred to inter and intra-generic species. Therefore, occurrence of such genetic combinations (co-existence) of drug resistance and virulence factors in *E. coli* may be more frequent in future due to the powerful selection pressure created by extensive and indiscriminate use of antibiotics particularly, in developing countries like India. Besides, transferable nature of plasmids and wide spread occurrence of transposons both on bacterial plasmids and chromosome will further enhance the accumulation, formation and spread of multi-virulent plasmids, resulting in many fold increase in the virulence

of the host bacterium. Such plasmids will also have an extra advantage to the isolates involved in intestinal (diarrhoea) and extra intestinal infections of man and animals. Because of their free transmission from man to animals and vice versa, this will still create very serious zoonotic problem.

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ANNEXURES

ANNEXURE I :

Sugar fermentation reactions of 194 strains of Escherichia coli

Strain numbers	Lactose	Arabi-nose	Xylose	Sorbi-tol	Rhamnose	Dulci-tol	Sucrose	Sali-chin	Raffi-nose	Adoni-tol	Inositol
2	3	4	5	6	7	8	9	10	11	12	13
01	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
02	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
03	AG	AG	AG	AG	AG	AG	-	-	-	-	-
04	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
05	AG	AG	AG	AG	AG	AG	AG	-	-	-	-
06	AG	AG	AG	AG	AG	-	AG	AG	AG	-	-
07	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
08	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
09	AG	AG	AG	AG	AG	-	AG	AG	AG	-	-
10	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
11	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
12	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
13	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
14	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
15	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
16	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
17	AG	AG	AG	AG	AG	AG	-	AG	-	-	-
18	AG	AG	AG	AG	AG	-	AG	-	AG	-	-
19	AG	AG	AG	AG	AG	-	AG	AG	AG	-	-
20	AG	AG	AG	AG	AG	-	AG	AG	AG	-	-
21	AG	AG	AG	AG	AG	-	AG	AG	AG	-	-
22	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
23	AG	AG	AG	AG	AG	-	-	-	AG	-	-
24	AG	AG	AG	AG	AG	-	AG	-	-	-	-
25	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
26	AG	AG	AG	AG	A	-	AG	-	-	-	-
27	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
28	AG	AG	AG	AG	A	AG	AG	-	AG	-	-
29	AG	AG	AG	AG	A	AG	AG	-	AG	-	-
30	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-

2	3	4	5	6	7	8	9	10	11	12	13
31	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
32	AG	AG	AG	–	AG	AG	AG	–	AG	–	–
33	AG	AG	AG	AG	AG	AG	A	A	A	–	–
34	AG	AG	AG	AG	A	AG	AG	A	A	–	–
35	AG	AG	AG	AG	AG	AG	AG	AG	A	–	–
36	AG	AG	AG	–	A	–	A	–	–	–	–
37	AG	AG	AG	AG	AG	AG	A	AG	AG	–	–
38	AG	AG	AG	AG	AG	–	A	A	AG	–	–
39	AG	AG	AG	AG	AG	–	–	–	AG	–	–
40	AG	AG	AG	A	–	–	–	–	–	–	–
41	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
42	AG	AG	AG	AG	AG	–	AG	–	AG	–	–
43	AG	AG	AG	AG	A	AG	–	A	AG	–	–
44	AG	AG	AG	AG	AG	–	AG	–	–	–	–
45	AG	AG	AG	AG	AG	–	–	–	AG	–	–
46	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
47	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
48	AG	AG	AG	AG	A	AG	AG	–	AG	–	–
49	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
50	AG	AG	AG	AG	A	AG	–	–	AG	–	–
51	AG	AG	AG	AG	AG	AG	–	AG	–	–	–
52	AG	AG	AG	AG	AG	AG	AG	AGL	AG	–	–
53	AG	AG	AG	AG	AG	AGL	AG	AG	AG	–	–
54	AG	AG	AG	AG	AG	AGL	AG	AG	AG	–	–
55	AG	AG	AG	AG	AG	AG	AG	AGL	AG	–	–
56	AG	AG	AG	AG	AG	AG	–	AGL	–	–	–
57	AG	AG	AG	AG	AG	AG	AG	AGL	AG	–	–
58	AG	AG	AG	AG	AG	–	–	AG	–	–	–
59	AG	AG	AG	AG	AG	AG	AL	AG	AG	–	–
60	AG	AG	AG	AG	AG	AGL	–	–	AG	–	–
61	AGL	AG	AG	AG	AG	–	–	–	–	–	–
62	AG	AG	AG	AG	AG	AGL	–	AG	–	–	–
63	A	A	A	–	AG	–	A	–	A	–	–
64	AG	AG	AG	AG	AG	–	AG	AGL	AG	–	–
65	AG	AG	AG	AG	AG	AG	AG	AGL	AG	–	–
66	AG	AG	AG	AG	AG	–	–	–	–	AG	–
67	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
68	AG	AG	AG	AG	AG	AG	–	AGL	–	–	–
69	AG	AG	AG	AG	AL	AG	AG	AG	AG	–	–
70	AG	AG	AG	AG	AG	–	–	AGL	–	AG	–
71	AG	AG	AG	AG	AG	AGL	–	AGL	–	–	–
72	AG	AG	AG	AG	AG	AG	–	AGL	–	–	–

2	3	4	5	6	7	8	9	10	11	12	13
73	AG	AG	AG	AG	AG	–	–	AGL	–	–	–
74	AG	AG	AG	AG	AG	AG	–	AGL	–	–	–
75	AG	AG	AG	AG	AG	AG	AGL	AG	–	–	–
76	AGL	AG	–	AG	ag	–	–	AGL	–	–	–
77	AG	AG	AG	AG	AG	AG	–	–	–	–	–
78	AG	AG	AG	AG	AG	AG	AG	AGL	AG	–	–
79	AG	AG	AG	AG	AG	AG	–	AGL	–	–	–
80	AG	AG	AG	AG	AG	AG	–	AGL	–	–	–
81	AG	AG	AG	AGL	AG	–	–	–	–	AG	–
82	AG	AG	AG	AG	AG	AG	A	–	A	–	–
83	AG	AG	AG	AG	AG	AG	AGL	–	AG	–	–
84	AG	AG	AG	AG	AG	AG	AGL	–	AG	–	–
85	AG	AG	AG	AG	AG	AG	AG	–	AGL	–	–
86	AG	AG	AG	AGL	AG	–	–	–	–	–	–
87	AG	AG	AG	AG	AG	AGL	–	–	–	–	–
88	AG	AG	AG	AG	AG	AG	AG	AGL	AGL	–	–
89	AG	AG	AG	AG	AG	–	–	AGL	–	–	–
90	AG	AG	AG	AG	AG	AG	AG	AGL	AG	–	–
91	AG	AG	AG	AG	AG	–	AG	AGL	AGL	–	–
92	–	AG	AG	AG	AG	–	AG	–	–	–	–
93	–	AG	AG	AG	AG	AG	AG	–	AG	–	–
94	AG	AG	AG	AG	AG	AG	AG	–	–	–	–
95	AG	AG	AG	AG	–	–	–	AG	–	AG	–
96	AG	AG	AG	AG	AG	–	–	–	–	AG	–
97	AG	AG	AG	AG	–	AG	AG	–	AG	–	–
98	AG	AG	AG	AG	AG	AG	AG	–	AG	–	–
99	AG	AG	AG	AG	AG	AG	AG	–	–	–	–
100	AG	AG	AG	AG	AG	AG	AG	AGL	AGL	–	–
101	AG	AG	AG	AG	AG	AG	–	–	–	–	–
102	AG	AG	AG	AG	AG	AG	–	–	–	–	–
103	AG	AG	AG	AG	AG	AG	–	AG	–	–	–
104	AG	AG	AG	AG	AG	–	–		–		

2	3	4	5	6	7	8	9	10	11	12	13
105	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
106	AL	AG	AG	-	AG	-	-	AGL	-	AG	-
107	AG	AG	AG	AG	AG	AG	-	AGL	-	-	-
108	AG	AG	AG	AG	AG	AG	-	AGL	-	-	-
109	AG	AG	AG	AG	AG	-	-	AG	-	-	-
110	AG	AG	AG	AG	AG	AG	-	AGL	AG	-	-
111	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
112	AG	AG	AG	AG	AG	AG	AG	-	AG	-	AL
113	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
114	AG	AG	AG	AG	AG		-	AG	-	-	-
115	AG	AG	AG	AG	AG	AGL	-	-	-	-	-
116	AG	AG	AG	AG	-	AGL	-	-	-	-	-
117	AG	AG	AG	AG	AG	AG	AGL	-	AG	-	-
118	AG	AG	AG	AG	A	AG	AGL	-	AG		-
119	AG	AG	AG	AG	A	AG	AG	-	AG	-	-
120	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
121	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
122	AG	AG	AG	AG	AG	AGL	-	-	-	-	-
123	AG	AG	AG	AG	-	-	AL	-	AG	-	-
124	-	AG	AG	AG		AG	AG	-	AG	-	-
125	AG	AG	AG	AG	AG	-	-	-	-	AG	-
126	AG	AG	AG	AG	AG	-	-	-	-	AG	-
127	-	-	AG	AG	-	-	AG	-	-	AG	-
128	AG		AG	AG	AG	AG	AG	-	AG	-	-
129	AG	AG	AG	AG	AG	-	-	AGL	-	-	-
130	AG	AG	AG	AG	AG	-	-	AGL	-	-	-
131	AG	AG	AG	AG	aG	AG	-	-	-	-	-
132	AG	AG	AG	AG	AG	AGL	-	-	-	-	-
133	AG	AG	AG	AG	AG	AGL	-	AGL	-	-	-
134	AG	AG	AG	AG	AG	AG	-	AGL	-	-	-
135	AG	AG	-	AG	-	AG	-	AGL	-	-	-
136	AG	AG	AG	-	AG	-	-	AGL	-	-	-
137	AG	AG	AG	AG	AG	AG	AGL	-	AG	-	-

2	3	4	5	6	7	8	9	10	11	12	13
138	AG	AG	AG	AG	AG	-	-	AGL	-	-	-
139	AG	AG	AG	AG	AG	AG	AGL	-	AG	-	-
140	AG	AG	AG	AG	AG	-	-	AGL	-	-	-
141	AG	AG	AG	AG	AG	-	AG	-	AG	-	-
142	AG	AG	AG	AG	AG	AG	-	-	-	-	-
143	AG	AG	AG	AG	AGL	AGL	AGL	-	AG	-	-
144	AG	AG	AG	AG	AG	-	-	AGL	-	-	-
145	AG	AG	AG	AG	AG	-	-	AGL	-	AG	-
146	AG	AG	AG	AG	AL	AG	-	AGL	-	-	-
147	AG	AG	AG	AG	AG	AGL	AGL	-	AG	-	-
148	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
149	AG	AG	AG	AG	AG	-	-	AG	-	AG	-
150	AG	AG	AG	AG	AG	-	-	AGL	-	-	-
151	AG	AG	AG	AG	AG	AG	A	AG	AG	-	-
152	AG	AG	AG	AG	AG	-	A	AG	A	-	-
153	AG	AG	AG	AG	AG	-	AG	-	AG	-	-
154	AG	AG	AG	AG	AG	-	AG	-	AG	-	-
155	AG	AG	AG	AG	AG	AG	A	AGL	A	-	-
156	AG	AG	AG	AG	AG	AGL	AG	AG	AG	-	-
157	AG	AG	AG	AG	AG	-	A	A	AG	-	-
158	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
159	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
160	AG	AG	AG	-	AG	AG	-	AG	AG	-	-
161	AG	AG	AG	AG	AG	AG	A	-	AG	-	-
162	AG	AG	AG	AG	-	-	-	-	AG	-	-
163	AG	AG	AG	AG	AG	AG	-	-	-	-	-
164	AG	AG	AG	AG	AG	-	A	-	-	-	-
165	AG	AG	AG	AG	-	-	AG	-	AG	-	-
166	AG	AG	AG	AG	AG	-	AG		AG	-	-
167	AG	AG	AG	AG	-	-	A	-	A	-	-
168	AG	AG	AG	AG	AG	-	AG	-	-	-	-
169	AG	AG	AG	AG	A	-	A	AG	AG	-	-
170	AG	AG	AG	AG	AG	AG	-	-	-	-	-
171	AG	AG	AG	AG	AG	AG	-	-	AG	-	-
172	AG	AG	AG	AG	AG	AG	-	-	AG	-	-

2	3	4	5	6	7	8	9	10	11	12	13
173	AG	AG	AG	AG	AG	AG	-	-	AG	-	-
174	AG	AG	AG	AG	AG	-	AG	A	-	-	-
175	AG	AG	AG	AG	AG	AG	AG	A	A	-	-
176	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
177	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
178	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
179	AG	AG	AG	AG	AG	-	AG	-	-	-	-
180	AG	AG	AG	-	AG	AG	AG	-	-	-	-
181	AG	AG	AG	AG	-	AG	A	-	A	-	-
182	AG	AG	AG	AG	AG	-	-	-	-	-	-
183	AG	AG	AG	AG	AG	-	A	-	-	-	-
184	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	-
185	AG	AG	AG	AG	AG	-	-	-	-	-	-
186	AG	AG	AG	AG	AG	-	-	-	AG	-	-
187	AG	AG	AG	AG	AG	AG	A	AG	A	-	-
188	AG	AG	AG	AG	AG	-	-	-	A	-	-
189	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-
190	AG	AG	AG	AG	AG	-	A	-	AG	-	-
191	AG	AG	AG	AG	AG	AG	-	AG	AG	-	-
192	AG	AG	AG	AG	AG	AG	AG	-	A	-	-
193	AG	AG	AG	AG	AD	-	A	-	A	-	-
194	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-

Abbreviations:

A= Acid production
AG= Acid and Gas production
AGL/AL= Delayed reaction beyond 72 hrs, producing acid or gas of both.
- = No fermentation of carbohydrate.

Annexure 2: Somatic antigen groups (O-serogroups) of 194 strains of Escherichia coli

O-serogroup	Strain no.	Total	O-serogroup	Strain no.	Total
O1	84,94,95	3	O25	48,91,154,160	4
O2	46,117,127,139,147	5	O32	122	1
O4	50	1	O35	55,67,75,76,89,89,92	6
O5	146	1	O36	41	1
O7	77,82	2	O38	62,121	2
O8	184	1	O43	135	1
O9	51,68,71,109,112,125	6	O44	151,153	2
O12	138	1	O45	73	1
015	39,52,60,64,100	5	O50	47	1
O17	66,99	2	O51	129	1
O20	59,101,110,126,134	5	O55	97	1
O22	90,98	2	O57	80	1
O23	61,120	2	O60	44,58,88,156	4
061	56,72,83	3	O106	144	1
O68	118,119,130,140	4	O113	107	1
O70	79,81	2	O115	57	1
O73	69,137,144	3	O121	150	1
O78	40	1	O128	141	1
O80	86	1	O143	136,145	2
O83	132	1	O145	115	1
O84	63,155,164	3	O147	131,133,148,149	4
O86	167	1	O149	85	1
O88	74,143	2	O154	70,114	2
O93	104,123	2	O156	113	1
O98	93,124	2	O158	128	1
O100	65	1	O159	87,102	2
O101	157	1			
O103	96,103,105,116	4	**Total typable strains**		112

Untyped Strains *

1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28, 29,30,31,32,33,34,35,36,37,38,42,43,45,49,53,54,78,106,108,111,152, 158,159,161,162,163,165,166,168,169,170,171,172,173,174,175,176,177, 178,179,180,181,182,183,185,186,187,188,189,190,191,192,193,194, — 82

*82 strains could not be typed with the available O-antisera at CRI Kasauli (India).

ANNEXURE 3: Minimum inhibitory concentrations (µg/ml) of antibacterial drugs against 194 strains of Escherichia coli

Strain number	Ampicillin Ap	Amoxycillin Ax	Tetracyc- Tc	Doxycycline Dx	Streptomycin Sm	Chloramphenicol Cm	Kanamycin Km	Nitrofurantoin Fd	Co-trimoxazole Co	Nalidixic Acid Nal	Norfloxacin Nx
01	3.125	3.125	1.56	0.8	12.5	1.56	12.5	12.5	6.25	3.125	0.2
02	200	200	200	100	12.5	400	6.25	6.25	12.5	1.56	0.2
03	3.125	6.25	400	400	12.5	1.56	12.50	6.25	12.50	1.56	0.2
04	800	800	0.80	0.80	400	100	6.25	6.25	12.5	1.56	0.2
05	1.56	1.56	0.8	0.8	6.25	0.8	6.25	6.25	12.5	0.4	0.1
06	3.125	3.125	1.56	1.56	12.5	3.125	12.50	12.5	12.5	3.125	0.1
07	400	400	100	100	800	400	12.50	12.5	6.25	3.125	0.1
08	3.125	6.25	400	200	12.5	3.125	6.25	6.25	12.5	3.125	0.1
09	12.5	12.5	1.56	1.56	6.25	1.56	6.25	12.5	12.50	1.56	0.1
10	12.5	12.5	1.56	1.56	12.5	1.56	12.5	12.5	6.25	1.56	0.1
11	25.0	25.00	200	12.5	12.5	100	6.25	12.50	25.00	1.56	0.2
12.	3200	3200	400	200	50.00	400	6.25	12.5	12.5	1.56	0.2
13.	25	1.56	1.56	1.56	12.5	1.56	6.25	12.5	12.5	1.56	0.2
14	6400	6400	1.56	0.8	25.00	3.125	6.25	12.5	100.00	3.125	0.1
15	12.50	3.125	3.125	1.56	12.5	1.56	6.25	12.5	25.00	3.125	0.1
16	3.125	3.125	12.5	12.56	12.5	1.56	1.56	12.5	12.5	3.125	0.2
17	1600	800	400	200	6.25	0.800	12.50	12.5	200	1.56	0.2
18	6400	6400	1.56	1.56	12.5	1.56	3.125	100	50.00	3.12	0.1
19	1600	1600	100	50	3.125	200	12.5	6.25	50.00	0.8	0.1
20	25.00	25.00	400	400	6400	400	6.25	25.00	100	3.12	0.3
21	200	200	200	200	12.50	200	25.0	25.00	3200	3.12	0.3
22	3.125	6.25	200	12.5	50.00	0.4	12.50	12.50	200	1.56	0.1
23	50	6.25	800	400	25.0	0.4	12.50	12.50	200	3.12	0.1
24	3.125	3.125	400	200	6.25	0.8	6.25	25.0	1600	3.12	0.1
25	3.125	3.125	3.125	1.56	100	0.8	12.5	12.50	6.25	3.12	0.05
26	6.25	1.56	800	400	12.50	400	3.125	12.50	800	6.25	0.05
27	12.5	6.25	1.56	0.8	6.25	0.4	12.5	12.5	12.5	3.12	0.05
28.	6.25	3.125	100	50	12.5	100	12.5	6.25	1600	1.56	0.3
29	1.56	1.56	12.50	12.50	6.25	12.5	6.25	6.25	12.50	6.25	0.3
30	6400	6400	400	400	6.25	400	6.25	25.0	1600	6.25	0.2

Strain No.	Ap	Ax	Tc	Dx	Sm	Cm	Km	Fd	Co	Nal	NY
31	3.12	1.56	1.56	0.8	12.5	0.80	12.50	12.50	12.50	3.12	0.1
32	3200	1600	100	3.12	200	1.56	3.12	6.25	12.50	3.12	0.1
33	3.12	1.56	3.12	1.56	12.5	1.56	3.12	12.5	12.50	6.25	0.2
34	1600	1600	3.12	1.56	12.5	1.56	6.25	6.25	12.5	1.56	0.2
35	3.12	0.80	3.12	1.56	6.25	1.56	12.50	12.5	6.25	1.56	0.2
36	6.25	3.12	3200	1600	12.5	400	12, 50	12.5	3200	6.25	0.3
37	3200	200	400	100	12.50	200	6.25	12.5	6.25	3.12	0.1
38	6400	6400	1600	800	80 ρ	200	6.25	50	3200	6.25	0.1
39	1600	800	400	100	12.5	200	50	12.5	12.5	1.56	0.1
40	12.5	3.12	400	50.00	200	200	50	6.25	12.5	1.56	0.1
41	100	12.5	200	2500	200	200	12.5	6.25	12.5	1.56	0.1
42	1600	1600	1.56	0.80	25.0	12.50	12.5	12.5	12.5	12.5	2.0
43	3.12	1.56	200	100	100	12.5	6.25	12.5	12.5	1.56	0.1
44	1600	1600	200	100	12.5	800	50.00	12.5	25.0	25.0	1.00
45	200	50	200	100	1600	200	12.5	12.5	12.5	1.56	0.1
46	1600	1600	100	12.5	3.12	100	12.50	12.5	12.5	12.50	2.00
47	100	50	12.5	3.12	6.25	3.12	12.5	12.5	12.5	1.56	0.05
48	3.12	1.56	400	200	1600	800	12.5	12.5	100	12.5	2.00
49	3.12	1.56	200	200	100	200	12.5	12.5	25.0	3.12	0.4
50	3.12	1.56	200	200	400	12.5	12.5	12.5	12.5	1.56	0.2
51	3.12	1.56	50	6.25	200	6.25	3.12	12.5	12.5	3.12	1.00
52	6.25	3.12	12.50	3.12	3.12	12.5	6.25	1.56	12.5	1.56	1.50
53	3.12	1.56	100	50.0	200	12.5	3.12	12.5	6.25	3.12	0.1
54	3.12	3.12	1.56	0.8	12.5	12.5	3.12	25.0	12.5	3.12	0.1
55	6.25	6.25	3.12	1.58	12.5	6.25	1.56	12.5	12.5	3.12	0.1
56	6.25	6.25	3.12	0.80	12.5	3.12	3.12	6.25	12.5	3.12	0.1
57	6.25	3.12	3.12	1.56	12.5	6.25	3.12	12.5	12.5	1.56	0.05
58	3.12	3.12	3.12	1.56	50	12.5	1.56	6.25	12.5	3.12	0.05
59	3.12	3.12	100	50	25	400	1.56	25.0	12.5	3.12	0.05
60	40 0	400	50	25	200	400	80 0	12.5	12.5	3.12	0.10
61	400	400	3.12	1.56	100	3.12	1.56	6.25	6.25	6.25	0.20
62	1600	1600	3.12	1.56	12.5	6.25	6.25	12.5	6.25	1.56	0.10
63	3.12	3.12	50	50	12.5	6.25	12.5	6.25	6.25	1.56	0.10
64	6.25	3.12	3.12	3.12	12.5	3.12	3.12	6.25	6.25	1.56	0.10
65.	6.25	6.25	3.12	3.12	12.50	6.25	3.12	12.5	6.25	3.12	0.50
66	6.25	6.25	25	25	200	3.12	1.56	3.12	12.5	1.56	0.05
67	3.12	1.56	3.12	1.56	12.50	6.25	1.56	6.25	12.5	3.12	0.1
68	3.12	1.56	25	25	200	3.12	3.12	12.5	12.5	1.56	0.1
69	6.25	3.12	12.5	12.5	200	12.5	1.56	12.5	25.0	1.56	0.05
70	3.12	3.12	50	50	12.5	3.12	1.56	6.25	100	1.56	0.05

Strain No.	Ap	Ax	Tc	Dx	Sm	Cm	Km	Fd	Co	Na	N·y 1·
71	6.25	3.12	100	100	200	6.25	1.56	12.5	25.0	3.12	0.05
72	25	25	12.5	6.25	12.5	6.25	12.5	12.5	12.5	3.12	0.025
73	25	25	12.5	6.25	100	6.25	3.12	12.5	6.25	3.12	0.1
74	3.12	3.12	3.12	1.56	200	3.12	1.56	12.5	6.25	6.25	0.1
75	6.25	3.12	6.25	1.56	12.5	6.25	3.12	6.25	12.5	3.12	0.05
76	1600	800	100	50.00	12.5	12.5	12.5	12.5	200	3.12	0.025
77	6.25	3.12	1.56	1.56	12.5	3.12	3.12	25	6.25	3.12	0.1
78	6.25	3.12	3.12	0.8	12.5	12.5	3.12	12.5	6.25	3.12	0.1
79	3.12	1.56	1.56	0.8	12.5	6.25	1.56	6.25	6.25	3.12	0.2
80	3.12	1.56	100	100	200	12.5	3.12	12.5	6.25	1.56	0.1
81	3.12	1.56	50	12.5	125	6.25	1.56	12.5	6.25	3.12	0.1
82	25	12.5	100	100	3200	400	3.12	12.5	12.5	3.12	0.1
83	6.25	3.12	3.12	1.56	12.5	6.25	1.56	25	6.25	3.12	0.1
84	6.25	3.12	3.12	1.56	12.50	3.12	3.12	12.5	12.5	3.12	0.2
85	1600	800	100	50	1600	400	1.56	12.5	12.5	3.12	0.1
86	6.25	3.12	100	50	200	6.25	3.12	12.5	12.6	3.12	0.1
87	1600	1600	50	12.5	200	100	1.56	6.25	6.25	1.56	0.05
88	6.25	6.25	3.12	1.56	12.5	6.25	3.12	12.5	6.25	3.12	0.1
89	6.25	6.25	3.12	1.56	12.5	3.12	1.56	6.25	6.25	3.12	0.1
90	3.12	1.56	1.56	0.8	12.5	6.25	3.12	6.25	12.5	1.56	0.05
91	6.25	3.12	3.12	0.8	12.5	3.12	1.56	6.25	12.5	3.12	0.1
92	3.12	1.56	1.56	1.56	50	6.25	1.56	3.12	12.5	3.12	0.1
93	6.25	1.56	12.5	12.50	12.50	12.5	3.12	6.25	2.5	3.12	0.1
94	3.12	1.56	50	50	12.50	1.56	3.12	12.5	12.5	1.56	0.05
95	1600	800	200	100	400	400	1.56	12.5	6.25	3.12	0.05
96	6.25	6.25	12.5	12.5	6.25	6.25	1.56	12.5	6.25	3.12	0.1
97	3.12	1.56	3.12	1.56	6.25	3.12	1.56	25	12.5	3.12	0.025
98	3.12	1.56	1.56	1.56	25	3.12	1.56	25	12.5	1.56	0.25
99	3.12	3.12	3.12	1.56	12.5	12.50	1.56	12.50	12.5	3.12	0.20
100	3.12	1.56	6.25	3.12	12.5	12.5	3.12	25	12.5	3.12	0.2
101	3.12	1.56	100	100	12.5	6.25	1.56	25	12.5	3.12	0.1
102	3.12	1.56	100	12.5	200	3.12	1.56	50	12.5	3.12	0.1
103	3.12	1.56	25	6.25	200	800	3.12	100	12.5	3.12	0.1
104	3.12	1.56	1.56	1.56	50	6.25	1.56	25	12.5	3.12	0.1
105	3.12	1.56	3.12	1.56	100	3.12	1.56	25	12.5	3.12	0.1

Strain No.	Ap	Ax	Tc	Dx	Sm	Cm	Km	Pd	Co	Na	Hy
106	3.12	1.56	1.56	0.8	12.5	6.25	1.56	12.5	12.5	3.12	0.2
107	12.5	6.25	50	25	200	3.12	1.56	12.5	6.25	3.12	0.1
108	800	6.25	50	25	400	200	1.56	25	6.25	1.56	0.2
109	800	800	200	200	400	1600	1.56	25	6.25	3.12	0.05
110	6.25	6.25	200	200	200	6.25	1.56	25	6.25	3.12	0.1
111	6.25	6.25	1.56	1.56	100	3.12	1.56	12.5	200	3.12	0.05
112	3.12	1.56	3.12	3.12	50	3.12	1.56	25	12.5	3.12	0.1
113	6.25	6.25	1.56	1.56	50	6.25	1.56	12.5	6.25	3.12	0.05
114	12.5	6.25	3.12	1.56	12.5	12.5	0.78	12.5	6.25	3.12	0.1
115	1600	1600	50	2500	400	800	1.56	100	12.5	3.12	0.2
116	3.12	3.12	50	1.56	400	800	1.56	25	12.5	3.12	0.2
117	6.25	6.25	1.56	1.56	12.5	3.12	0.78	25	12.5	1.56	0.05
118	12.5	6.25	200	50	3200	12.50	1.56	25	800	3.12	0.05
119	800	800	200	200	3200	1600	3.12	25	6.25	1.56	0.1
120	3.12	3.12	100	12.5	6.25	6.25	1.56	12.5	6.25	1.56	0.1
121	3.12	3.12	25	12.5	1600	400	3.12	25	12.5	3.12	0.1
122	800	800	200	100	3200	800	12.5	12.5	400	3.12	0.1
123	3.12	3.12	25	12.5	800	800	1.56	12.5	6.25	1.56	0.1
124	3.12	3.12	50	12.5	12.5	3.12	1.56	25	12.5	3.12	0.1
125	6.25	3.12	100	50	200	6.25	3.12	25	12.5	3.12	0.1
126	12.5	6.25	3.12	1.56	25	3.12	1.56	25	12.5	3.12	0.2
127	25.	12.50	25	12.5	200	100	3.12	400	12.5	3.12	0.05
128	6.25	3.12	50	25	800	12.5	1.56	25	6.25	1.56	0.1
129	1.56	0.8	12.5	12.5	12.5	3.12	3.12	25	6.25	1.56	0.05
130	3.12	1.56	1.56	0.8	12.5	3.12	1.56	12.5	12.5	3.12	0.05
131	3.12	1.56	3.12	0.8	12.5	6.25	3.12	25	12.5	6.25	0.05
132	1600	1600	100	100	800	1600	1.56	100	800	3.12	0.1
133	3.12	1.56	1.56	1.56	50	6.25	1.56	12.5	200	3.12	0.05
134	3.12	1.56	1.56	1.56	100	3.12	1.56	12.5	12.5	3.12	0.05
135	3.12	1.56	3.12	1.56	12.5	6.25	3.12	25	6.25	1.56	0.05
136	1.56	0.8	0.8	0.8	12.5	3.12	1.56	12.5	6.25	0.78	0.05
137	3.12	1.56	12.5	12.5	12.5	3.12	1.56	25	12.5	3.12	0.025
138	1.56	1,56	0.8	0.8	100	6.25	1.56	12.5	12.5	1.56	0.02
139	3.12	12.5	3.12	12.5	400	6.25	1.56	25	6.25	3.12	0.02
140	1.56	1.56	0.8	0.8	12.5	3.12	1.56	6.25	6.25	3.12	0.05
141	3.12	1.56	1.56	1.56	12.5	12.5	1.56	25	6.25	1.56	0.05
142	6.25	3.12	12.5	12.5	12.5	12.5	1.56	12.5	6.25	1.56	0.05
143	6.25	12.5	3.12	3.12	6.25	1.56	3.13	25	100	3.12	0.05
144	3.12	3.12	1.56	1.56	100	3.12	1.56	25	12.5	3.12	0.1
145	3.12	3.12	1.56	1.56	12.5	6.25	1.56	25	12.5	3.12	0.05

Strain No.	Ap	Ax	Tc	Dx	Sm	Cm	Km	Fd	Co	Nal	No
146.	400	200	100	100	100	400	1.56	12.5	12.5	1.56	0.05
147	6.25	6.25	3.12	3.12	50	12.5	1.56	25	12.5	3.12	0.01
148	3.12	3.12	100	12.5	12.5	12.5	1.56	25	100	3.12	0.1
149	1600	12.5	200	12.5	12.5	1.56	1.56	25	12.5	3.12	0.40
150	3.12	3.12	6.25	12.5	6.25	3.12	3.12	25	12.5	3.12	0.05
151	3.12	1.56	6.25	3.12	12.5	6.25	1.56	12.5	12.5	6.25	0.2
152	3.12	3.12	1.56	0.8	25.0	6.25	25.0	12.5	12.5	6.25	0.2
153	3.12	1.56	12.5	6.25	12.5	6.25	25.0	12.5	6.25	3.12	0.1
154	3.12	1.56	12.5	6.25	12.5	3.12	6.25	12.5	12.5	1.56	0.01
155	3.12	1.56	1.56	0.8	12.5	12.5	12.5	12.5	12.5	3.12	0.1
156	100	3.12	100	12.5	12.5	3.12	1.56	6.25	12.5	1.56	0.1
157	3.12	1.56	12.5	6.25	12.5	6.25	25.0	12.5	12.5	3.12	0.3
158	3.12	1.56	200	100	12.5	6.25	12.5	12.5	12.5	3.12	0.1
159	6.25	6.25	100	50	6.25	1.56	50	6.25	12.5	6.25	0.05
160	12.5	3.12	12.5	3.12	6.25	3.12	12.5	6.25	6.25	6.25	0.1
161	6.25	1.56	6.25	1.56	12.5	1.56	12.5	25	12.5	1.56	0.025
162	6.25	1.56	12.5	3.12	12.5	0.8	12.5	12.5	12.5	1.56	0.025
163	6.25	1.56	100	50	200	0.8	6.25	12.5	12.5	3.12	0.025
164	6.25	1.56	100	50	100	0.8	100	25.0	6.25	3.12	0.05
165	6.25	3.12	6.25	1.56	12.5	1.56	6.25	12.5	6.25	3.12	0.05
166	3.12	1.56	6.25	3.12	12.5	6.25	12.5	12.5	6.25	1.56	0.1
167	3.12	1.56	1.56	1.56	6.25	6.25	12.5	6.25	6.25	6.25	0.2
168	3.12	1.56	200	100	6.25	3.12	12.5	6.25	12.5	1.56	0.025
169	1.56	1.56	400	200	12.5	1.56	12.5	6.25	6.25	1.56	0.1
170	3.12	3.12	1.56	0.8	12.5	0.8	12.5	12.5	12.5	1.56	0.1
171	1.56	1.56	3.12	1.56	6.25	1.56	6.25	6.25	12.5	1.56	0.05
172	1.56	1.56	3.12	1.56	6.25	1.56	6.25	12.5	25.0	3.12	0.025
173	3.12	1.56	1.56	0.8	12.5	1.56	12.5	12.5	12.5	1.56	0.05
174	1.56	1.56	3.12	1.56	6.25	1.56	12.5	12.5	12.5	1.56	0.1
175	6.25	3.12	200	200	3.12	3.12	6.25	12.5	12.5	1.56	0.2
176	3.12	3.12	12.5	6.25	1.56	1.56	6.25	6.25	6.25	3.12	0.1
177	1.56	1.56	12.5	6.25	1.56	3.12	6.25	12.5	12.5	1.56	0.1
178	3.12	1.56	12.5	6.25	12.5	6.25	6.25	12.5	12.5	3.12	0.05
179	3.12	1.56	3.12	1.56	12.5	1.56	6.25	12.5	12.5	1.56	0.05
180	6.25	1.56	6.25	3.12	12.5	1.56	6.25	12.5	12.5	1.56	0.1
181	12.5	3.12	12.5	6.25	12.5	3.12	12.5	25	12.5	1.56	0.2
182	1.56	0.8	6.25	1.56	6.25	3.12	12.5	12.5	12.5	3.12	0.1
183	3.12	1.56	3.12	1.56	12.5	12.5	12.5	12.5	6.25	1.56	0.2
184	6.25	1.56	1.56	1.56	3.12	6.25	6.25	12.5	6.25	6.25	0.2

Strain No.	Ap	Ax	Tc	Dx	Sm	Cm	Km	Fd	Co	Na.	Nγ
185	12.5	6.25	6.25	1.56	12.5	0.78	12.5	12.5	12.5	3.125	0.025
186	12.5	6.25	3.12	1.56	12.5	1.56	12.5	12.5	6.25	6.25	0.025
187	12.5	3.12	3.12	1.56	12.5	0.78	12.5	25.0	12.5	6.25	0.1
188	50	12.5	6.25	3.12	12.5	0.78	12.5	12.5	12.5	6.25	0.1
189	12.5	6.25	3.12	3.12	12.5	1.56	12.5	12.5	12.5	3.12	0.025
190	12.5	6.25	3.12	1.56	12.5	1.56	12.5	25	12.5	3.12	0.2
191	12.5	6.25	6.25	1.56	12.5	1.56	12.5	25	12.5	3.12	0.2
192	12.5	6.25	1.56	0.78	12.5	3.12	6.25	12.5	6.25	6.25	0.3
193	6400	6400	6.25	1.56	12.5	6.25	12.5	25	3200	3.12	0.1
194	6400	6400	800	400	1600	3.12	3.12	12.5	1600	3.12	0.1
E. coli B	6.25	6.25	6.25	3.125	6.25	3.125	6.25	12.5	12/5	3:125	0.1

LIST OF PUBLICATIONS

Papers published/accepted for publication :

1. J.N.S. Yadava, **Iqbal Ahmad,** Shamim Ahmad and K.R. Bhardwaj (1992). Genetic stability of R-plasmids among pathogenic isolates of *Escherichia coli* of man and animal origin. *Indian Veterinary Medical Journal.* **16 (4) :** 260-67.

2. Kavita Gupta, **Iqbal Ahmad** and J.N.S. Yadava (1993). Isolation, purification and characterization of heat labile enterotoxin form an enterotoxigenic strain of *Escherichia coli.* Proceeding of CSIR India (Golden jubilee symposium on tropical diseases, molecular biology and control strategies held at CDRI, Lucknow 17-20 February, 1992). In press.

3. **Iqbal Ahmad,** Shamim Ahmad and J.N.S. Yadava (1993). High level of transferable resistance among *Escherichia. coli. Indian Journal of Animal Sciences.* **64** : (In press).

4. **Iqbal Ahmad,** Shamim Ahmad, K.R. Bhardwaj and J.N.S. Yadava (1994). Prevalence of enterotoxigenic *Escherichia coli* strains among diarrhoeal isolates of laboratory animals. *Laboratory Animals India* (In press).

Papers communicated for publication :

1. **Iqbal Ahmad,** Shamim Ahmad and J.N.S.Yadava. Elimination of R-factors among *E.coli* strains with special reference to norfloxacin. *Journal of Antimicrobial Chemotherapy.*

2. Shamim Ahmad and **Iqbal Ahmad.** Evaluation of ocular mycotic pathogens among diverse origin. *Indian Journal of Ophthalmology.*

3. **Iqbal Ahmad,** Shamim Ahmad and J.N.S.Yadava. Plasmid encoded Virulence factors of *E. coli* strains : A correlation study. *Journal of Tropical Medicine and Hygiene.*

Papers presented/accepted in symposia/conferences :

1. **Iqbal Ahmad,** M.A.Choudhary, J.N.S.Yadava and Shamim Ahmad (1990). Studies on the emergence of transferable antibiotic resistance among pathogenic strains of *E.coli.* Paper presented (Oral session) in *National Symposium on Frontiers in Modern Biology,* Dec. 1-3, 1990, Jamia Milia Islamia, New Delhi.

2. **Iqbal Ahmad**, S.Ahmad, K.Gupta and J.N.S.Yadava (1991). Norfloxacin: A better curing agent for R-plasmids among *Escherichia coli.* Paper presented in *60th Annual Meeting of Society of Biological Chemists (India)* Dec. 27-30, Calcutta.

3. **Iqbal Ahmad**, S.Ahmad and J.N.S.Yadava (1991). Emergence of multiple drug resistance and their affinity with virulence factors among *Escherichia coli* strains of clinical origin. Paper presented in *60th Annual Meeting of Society of Biological Chemists (India)* December 27-30, Calcutta.

4. S.L.Narsimhan, J.N.S.Yadava, K.Gupta and **Iqbal Ahmad** (1992). Emergence of multiple drug resistance among pathogenic isolates of *Shigella* spp. Paper presented in *32nd Conference of Association of Microbiologists India.* Jan. 10-12, M.K.U., Madurai, Tamil Nadu.

5. **Iqbal Ahmad**, S. Ahmad and J.N.S. Yadava (1993). Genetic transfer of enterotoxigenicity and antibacterial resistance among *E. coli* strains. Paper accepted in *Annual Meeting of Society of Biological Chemists (India)* Dec. 1993 Madurai, Tamil Nadu.

6. **Iqbal Ahmad**, Shamim Ahmad and J.N.S.Yadava (1994). Plasmid encoded production of colicin and their compatibility with drug resistance determinants among *Escherichia coli* strains of man and animals origin. Paper presented in the *81st Annual Meeting of Indian Science Congress* on 3rd -6th Jan. 1994. held at Jaipur (India)

7. **Iqbal Ahmad**, Shamim Ahmad, J.N.S.Yadava and N.B.Singh (1994). Plasmids influencing virulence and pathogenicity of *Escherichia coli.* Paper accepted for presentation in the *XIth National Congress of Parasitology (India)* on Feb. 22nd - 24th 1994, at Udaipur, Rajasthan.